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RESEARCH REPORT

Di-sulfated Keratan Sulfate as a Novel Biomarker for Mucopolysaccharidosis II, IVA, and IVB

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Abstract Keratan sulfate (KS) is a storage material in mucopolysaccharidosis IV (MPS IV). However, no detailed analysis has been reported on subclasses of KS: mono-sulfated KS and di-sulfated KS. We established a novel method to distinguish and quantify mono- and di-sulfated KS using liquid chromatography–tandem mass spectrometry and measured both KS levels in various specimens.

Di-sulfated KS was dominant in shark cartilage and rat serum, while mono-sulfated KS was dominant in bovine cornea and human serum. Levels of both mono- and di-sulfated KS varied with age in the blood and urine from control subjects and patients with MPS II and IVA. The mean levels of both forms of KS in the plasma/serum from patients with MPS II, IVA, and IVB were elevated compared with that in age-matched controls. Di-sulfated KS provided more significant difference between MPS IVA and the age-matched controls than mono-sulfated KS. The ratio of di-sulfated KS to total KS in plasma/serum increased with age in control subjects and patients with MPS II but was age independent in MPS IVA patients. Consequently, this ratio can discriminate younger MPS IVA patients from controls. Levels of mono- and di-sulfated KS in urine of MPS IVA and IVB patients were all higher than age-matched controls for all ages studied.

In conclusion, the level of di-sulfated KS and its ratio to total KS can distinguish control subjects from patients with MPS II, IVA, and IVB, indicating that di-sulfated KS may be a novel biomarker for these disorders.

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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Abbreviations

Cre	Creatinine
DBS	Dried blood spot
ECM	Extracellular matrix
GAG	Glycosaminoglycans
Gal	D-galactose
GALNS	*N*-acetylgalactosamine-6-sulfate sulfatase
GlcNAc	*N*-acetyl-D-glucosamine
KS	Keratan sulfate

LC-MS/MS	High-performance liquid chromatography–tandem mass spectrometry
MPS	Mucopolysaccharidoses
NBS	Newborn screening
β-Gal	β-galactosidase

Introduction

Keratan sulfate (KS), a linear polymer glycosaminoglycan (GAG), is composed of alternating D-galactose (Gal) and *N*-acetyl-D-glucosamine (GlcNAc) residues linked to β1 → 4 and β1 → 3, respectively. KS attaches to core proteins including aggrecan, luminican, keratocan, mimecan, and fibromodulin with fucose and sialic acid and is distributed in the articular cartilage, cornea, brain, and various endothelial cells (Funderburgh 2000). KS is almost always sulfated on C (6) of GlcNAc, while C (6) of Gal is sulfated to a variable extent, depending on the tissue and age (Bhavanandan and Meyer 1968). KS chains of fibromodulin in articular cartilage are highly sulfated compared to that in the cornea (Lauder et al. 1997; Nieduszynski et al. 1990), and the levels of sulfation in cornea and cartilage increase during normal aging (Liles et al. 2010; Brown et al. 1998).

Mucopolysaccharidosis IV includes two subtypes, mucopolysaccharidosis IVA and IVB (MPS IVA, Morquio A syndrome; MPS IVB, Morquio B syndrome), caused by the deficiency of *N*-acetylgalactosamine-6-sulfate sulfatase (GALNS) and β-galactosidase (β-gal), respectively. Either enzyme deficiency results in accumulation of undegraded KS especially in cells and extracellular matrix (ECM) of cartilage and cornea. Patients with a classic (severe) form of MPS IVA have a unique systemic skeletal dysplasia including short-trunk dwarfism, kyphoscoliosis, coxa valga, odontoid hypoplasia, abnormal gait, joint mobility problems, restriction of chest wall movement, and a life span of 20–30 years if an appropriate orthopedic intervention is unavailable. Patients with an attenuated form have a milder skeletal involvement, and most have normal life span (Dũng et al. 2013; Yasuda et al. 2013; Tomatsu et al. 2011, 2012a, 2013a, b; Northover et al. 1996; Montaño et al. 2007, 2008; Suzuki et al. 2001; Hendriksz et al. 2013; Möllmann et al. 2013; Harmatz et al. 2013). Patients with MPS IVB show a milder phenotype of skeletal dysplasia than patients with the severe form of MPS IVA. Consequently, clinical features of MPS IVB are similar to those in patients with an attenuated form of MPS IVA.

We have established a reproducible, sensitive, and specific assay technique to measure KS in blood, urine, and dried blood spot (DBS) specimens using liquid chromatography–tandem mass spectrometry (LC-MS/MS) (Tomatsu et al. 2010a, b, 2013a; Oguma et al. 2007a, b; Hintze et al. 2011). Similar methods have been validated in another laboratory (Martell et al. 2011). KS levels in blood and urine in patients with MPS IVA are associated with age and clinical severity (Tomatsu et al. 2010c) and are decreased with enzyme replacement therapy (ERT) on mouse model and bone marrow transplantation (BMT) in a patient with MPS IVA (Tomatsu et al. 2008; Chinen et al. 2014). Thus, KS levels in the blood and/or urine should be a suitable biomarker for early diagnosis, screening, clinical severity, and therapeutic efficacy in MPS IVA (Tomatsu et al. 2008, 2010c, 2013a). However, there is significant overlap of KS levels between age-matched controls and patients with MPS IVA, especially in patients older than 10 years of age, suggesting that better biomarkers or methodology for MPS IVA are needed (Tomatsu et al. 2010c). Blood KS level was also elevated in patients with MPS II and MPS IVB, resulting in difficulty to separate MPS IVA from MPS II and MPS IVB or other types of MPS by total KS (Tomatsu et al. 2005, 2010c). Patients with MPS IVA and IVB have a deficiency of the enzyme that directly involves KS metabolism, N-acetylgalactosamine-6-sulfate sulfatase (GALNS) and β-galactosidase, respectively. Therefore, elevation of KS in the blood and urine of these types of MPS is natural; however, it is unexpected that patients with other types of MPS, in which responsible enzymes do not directly involve the catabolic pathway of KS, have the elevation of KS in the blood and urine as well.

GALNS removes the sulfate at C(6) in Gal of the di-sulfated KS, Gal(6S)β1 → 4GlcNAc(6S), producing mono-sulfated KS, Galβ1 → 4GlcNAc(6S). Our previous results showed that the proportion of di-sulfated KS in total KS in the patients with MPS IVA is higher than that in the control subjects (Tomatsu et al. 2010c); but the low sensitivity and specificity of the assay to detect di-sulfated KS limited further studies. Improvement of the assay method to separate di-sulfated KS and mono-sulfated KS was needed to distinguish MPS IVA, control subjects, and other types of MPS precisely.

In this study, we have determined di-sulfated KS levels and the proportion of di-sulfated KS in total KS in the blood and urine of control subjects and patients with MPS II, IVA, and IVB by using a novel LC-MS/MS method. We have compared its feasibility of di-sulfated KS with mono-sulfated KS as a biomarker for MPS.

Materials and Methods

Materials

Polymer KS

Two purified polymer KS samples were supplied by Seikagaku Co. (Tokyo, Japan). One originated from bovine cornea (KS-I), and the other, from shark cartilage (KSP). The ratio of sulfation in these two polymer KS samples was investigated in comparison with human dried blood spot (DBS), serum and urine, and rat serum.

Subjects

Blood specimens (plasma/serum) were collected from 58 patients with MPS II (phenotype: severe, 47; attenuated, 11), 35 patients with MPS IVA (phenotype: severe, 32; undefined, 3), and five patients with MPS IVB (all attenuated) with informed consent. Urine specimens were collected from 14 patients with MPS IVA (all severe) and five patients with MPS IVB (all attenuated) with informed consent. Information of age and clinical phenotype of each patient was obtained. Plasma/serum and urine samples were similarly obtained from 144 and 44 healthy controls, respectively. The diagnosis of MPS II, MPS IVA, and MPS IVB was made on the basis of enzyme activity (iduronate-2-sulfatase, GALNS, and β-gal, respectively) being less than 5% of levels found in normal plasma, leukocytes, or fibroblasts. Clinical severity for patients with MPS IVA was classified based on patient age and height, as described previously (Montaño et al. 2008; Tomatsu et al. 2012b). The phenotype of patients with MPS IVB was determined using the same classification. Clinical severity for patients with MPS II was classified based according to CNS involvement (Rozdzynska et al. 2011). In previous experiments by LC-MS/MS, we confirmed that there is no difference of GAG value in specificity and sensitivity between plasma and serum (Oguma et al. 2007a, b; Tomatsu et al. 2010b, c).

Enzymes and Standard

Keratanase II from Seikagaku Co. was used to digest "polymer" KS to disaccharides. Keratanase II can digest polymers that contain either mono-sulfated KS, Galβ1 → 4 GlcNAc(6S), or di-sulfated KS, Gal(6S)β1 → 4GlcNAc (6S). Gel permeation chromatography (GPC)-HPLC study showed that 60 % of KS-I was digested with keratanase II (Tomatsu et al. 2013a, 2014). Di-sulfated KS (L4; 20 μg/ml), polymer KS (20 μg/ml), and chondrosine for internal standard (IS) (1 mg/ml) supplied by Seikagaku Co. were prepared separately in ddH_2O. Standard working solutions of KS-I (0.1, 0.2, 1.0, 2.0, and 10.0 μg/ml) and IS solution (5 μg/ml) were prepared.

Keratanase II produced in Seikagaku Co. will be available upon request.

Methods

Sample Preparation

Plasma/serum, urine specimens, and standards were prepared as follows: Ten micro liter of each plasma/serum and urine sample and 90 μl of 50 mM Tris–hydrochloric acid buffer (pH 7.0) were placed in wells of AcroPrep™ Advance 96-Well Filter Plates that have Ultrafiltration Omega 10 K membrane filters (PALL Corporation, NY, USA). The filter plates were placed on the receiver and centrifuged at 2,000 g for 15 min to remove free disaccharides. The membrane plates were transferred to a fresh receiver plate. Standards were added to unused wells of the filter plate. Ten micro liter of IS solution (5 μg/ml), 80 μl of 50 mM Tris–hydrochloric acid buffer (pH 7.0), and 10 μl of keratanase II (2 mU per sample) were added to each filter well. The plate was incubated in a water bath at 37°C for 15 h and centrifuged at 2,000 g for 15 min. The receiver plate containing disaccharides was stored at −20°C until injection to LC-MS/MS.

Apparatus

The chromatographic system consisted of 1260 Infinity Degasser, binary pump, auto-injector, thermostatted column compartment, and 1290 Infinity Thermostat (Agilent Technologies, Palo Alto, CA, USA) and a Hypercarb column (2.0 mm i.d. 50 mm, 5 μm, Thermo Electron, USA). The column temperature was kept at 60°C. The mobile phase was a gradient elution from 0.025% ammonia to 90% acetonitrile in 0.025% ammonia. The gradient condition was programmed as follows: The initial composition of 0% acetonitrile was kept for 0.1 min, linearly modified to 30% over 1.8 min, maintained at 30% for 0.3 min, returned to 0% over 0.01 min, and finally maintained at 0% for 2.5 min. The flow rate was 0.6 ml/min. The 6460 Triple Quad mass spectrometer (Agilent Technologies) was operated in the negative ion detection mode with thermal gradient focusing electrospray ionization (Agilent Jet Stream technology, AJS). The parameters of Jet Stream technology were as follows: drying gas temperature, 350°C; drying gas flow, 11 l/min; nebulizer pressure, 35 psi; sheath gas temperature, 400°C; sheath gas flow, 11 l/min; capillary voltage, 4,000 V; and nozzle voltage, 2,000 V. A *m/z* 462 precursor ion and *m/z* 97 product ion were used to detect and quantify the mono-sulfated KS, and a *m/z* 542 precursor ion and *m/z* 462

product ion were used to detect and quantify the di-sulfated KS (Zhang et al. 2005). A *m/z* 354.29 precursor ion and *m/z* 193.1 product ion were used to detect the IS. Peak areas for all components were integrated automatically using QQQ Quantitative Analysis software (Agilent Technologies), and peak area ratios (area of analytes/area of IS) were plotted against concentration by weighted linear regression. Raw data of LC-MS/MS were automatically preserved. The concentration of each disaccharide was calculated using QQQ Quantitative Analysis software. The data of urine samples were corrected by creatinine (Cre) levels with Creatinine (urinary) Colorimetric Assay Kit (Cayman Chem. MI, USA).

Method Validation

The recoveries of analytes were determined by adding di-sulfated KS to control serum, comparing the control serum without standard di-sulfated KS. Intraday precision evaluated as coefficient of variation (CV) was determined by replicate analyses ($n = 5$) of three different control specimens (serum, plasma, and urine). Interday precision was determined by replicate analyses ($n = 5$) of three different serum specimens (serum, plasma, and urine) on three separate days.

The selectivity of the assay was investigated by processing and analyzing five independent samples by the procedure described above without enzymatic digestion. Calibration curves were constructed by plotting the peak area ratio of the analytes to IS against the concentration of the analytes. Each calibration curve consisted of seven calibration points.

Statistical Analysis

Patients were grouped by age as follows: 0 to <3years (years), 3 to <5 years, 5 to <10 years, 10 to <15 years, 15 to <20 years, and 20 years and above. Mono-sulfated and di-sulfated KS value (μg/ml) as well as proportion of di-sulfated KS in serum/plasma and urine was summarized by age groups and patient groups (control, MPS II, MPS IVA, and MPS IVB). Data were summarized using mean and standard deviation (SD). Age-adjusted standardized values of mono-sulfated and di-sulfated KS were calculated for MPS IVA patients. Corresponding concentration of control subjects was used as a reference for standardization. Specifically, for the age-matched group, standardized mono-sulfated KS of MPS IVA patients = (mono-sulfated KS for patients in the age-matched group of MPS IVA − mean mono-sulfated KS for control subjects in the age-matched group)/(SD of mono-sulfated KS for control subjects in the age-matched group). The mean age-adjusted standardized mono- and di-sulfated KS of MPS IVA patients was compared using *t*-test. In addition, numbers and percentages of MPS IVA patients with mono- and di-sulfated KS levels in serum/plasma and urine more than 2 and 3 SD above the mean of age-matched controls were estimated. Chi-square test and logistic regression analysis were performed to compare the proportion of MPS IVA patients with mono- and di-sulfated KS more than 2 and 3 SD above the mean of age-matched controls. The odds ratio (OR) and *p* value are presented. Test and model assumptions were checked before statistical analyses. All tests were two-tailed at the level of significance of 0.05. Analysis was performed using SPSS for Windows (version 22.0, IBM, Chicago, IL, USA).

Results

Sulfation Pattern of KS

The peak of di-sulfated KS eluted at 3.22 min and was clearly detected by MRM conditions of *m/z* 542 of the precursor ion and *m/z* 462 of product ion (Fig. 1a). It was also detected by multiple reaction monitoring (MRM) conditions of *m/z* 462 of the precursor ion and *m/z* 97 of product ion, presumably due to loss of sulfate in the fragmentor. After digestion of KS-I (bovine cornea) by keratanase II, a major peak of mono-sulfated KS (*m/z* 462 precursor, *m/z* 97 product) eluted at 3.06 min, and a minor peak of di-sulfated KS eluted at 3.22 min (Fig. 1b). After digestion of KPS (shark cartilage), only the di-sulfated KS peak was observed (Fig. 1c). These sulfation patterns of KS-I and KSP were confirmed by using GPC-HPLC. GPC analysis also showed that the proportion of di-sulfated KS vs. mono-sulfated KS in KS-I was 42% vs. 58 , while KPS contained nearly 100% of di-sulfated KS (data not shown). Thus, the results by LC-MS/MS were consistent with those by GPC-HPLC.

In human serum specimens from normal control subjects digested by keratanase II, two peaks corresponding to mono- and di-sulfated KS were observed (Fig. 1d). The ratio of forms of sulfated KS was similar to that in bovine cornea. Serum sample from control rat showed a different pattern compared to human serum sample. Total level of KS in rat serum was much lower than that in human serum and the major form was di-sulfated KS (Fig. 1e). A human urine sample also showed the presence of mono- and di-sulfated KS with a higher proportion of di-sulfated KS than seen in human serum (Fig. 1f). In human DBS, two peaks corresponding to mono- and di-sulfated KS were detected, and the ratio of forms of sulfated KS was similar to that in human serum (Fig. 1g). These findings suggest that the sulfation pattern of KS depends on the tissue, species, and specimen analyzed.

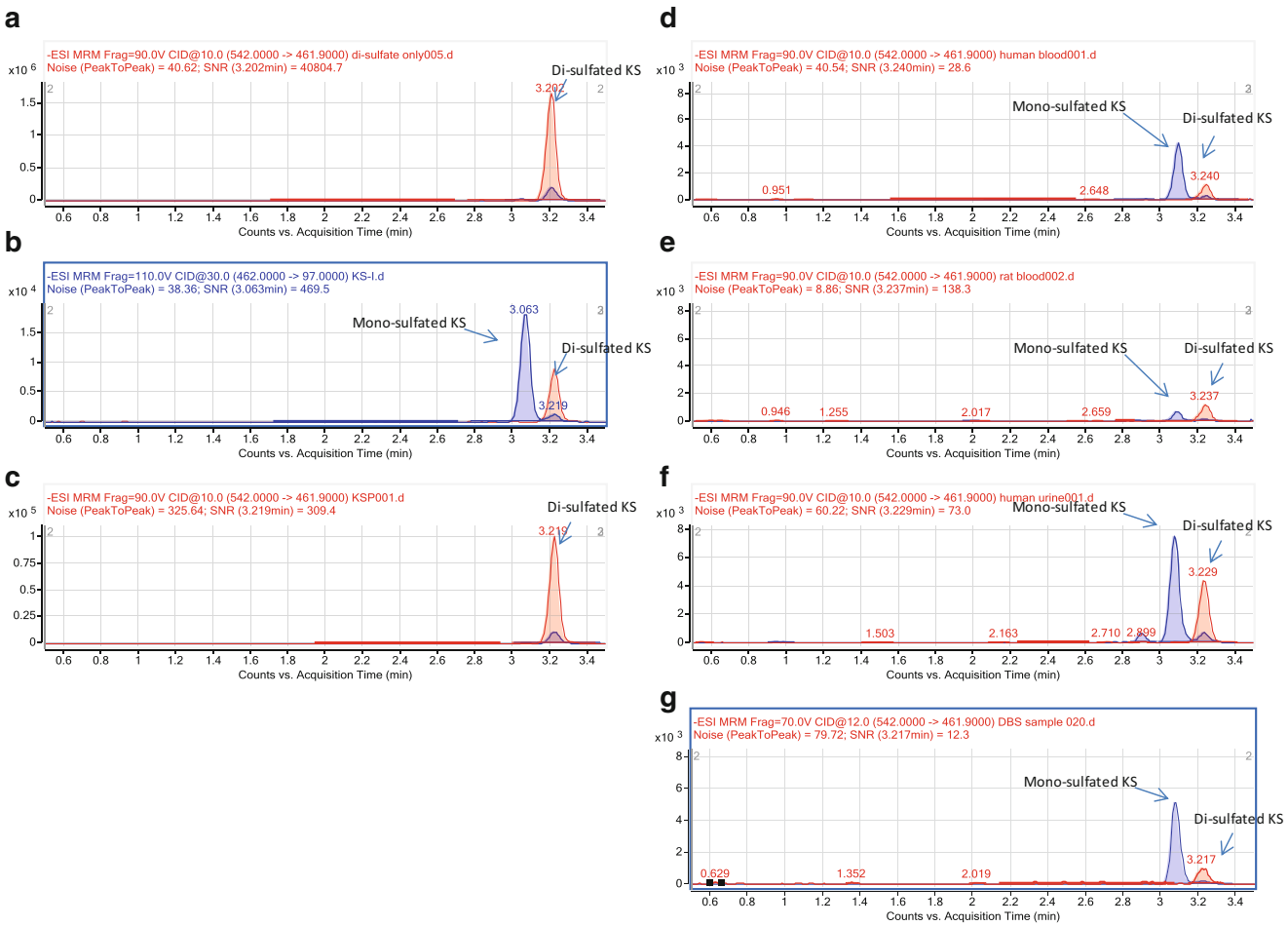

Fig. 1 Chromatogram for KS after digestion of keratanase II. (**a**) Di-sulfated KS standard. (**b**) KS-I: bovine cornea. (**c**) KSP: shark cartilage. (**d**) Human serum. (**e**) Rat serum. (**f**) Human urine. (**g**) Human dried blood spot (DBS). *Blue line* showed the MRM condition as *m/z* 462 precursor ion and *m/z* 97 product ion, which detected both mono- and di-sulfated KS. Red line showed the MRM condition as *m/z* 542 precursor ion and *m/z* 462 product ion, which detected di-sulfated KS only

Calibration Curves

Calibration curves for both of mono- and di-sulfated KS obtained on five separate days were linear over the concentration ranges of 0.1–10 µg/ml. The correlation coefficients (*r*) were not less than 0.99.

Precision and Accuracy

The mean recoveries of di-sulfated KS in control serum were 91.2% and 101.5% at concentration of 5.0 and 2.5 µg/ml, respectively. Mean recoveries of mono-sulfated KS in control serum are more than 87% at concentrations of 5.0 and 2.5 µg/ml (Oguma et al. 2007a, b). Results of intra- and inter-assay precision for mono- and di-sulfated KS in control serum are as follows: The intra-assay precision values/coefficient of variation (CV) determined from the analysis of mono- and di-sulfated KS are less than 6.8 and 14.6% in serum, 5.1 and 9.4% in plasma, and 14.1 and 12.9% in urine, respectively. The inter-assay precision values/CVs for these disaccharides are less than 6.5 and 14.3% in serum, 6.7 and 8.2% in plasma, and 12.3 and 15.8% in urine, respectively. These results demonstrate the reproducibility and accuracy of the method.

Analysis of Mono- and Di-sulfated KS

Blood Mono-sulfated KS: Galβ1 → 4GlcNAc(6S)

The mono-sulfated KS values for the plasma/serum samples from 58 MPS II patients (average age 11.5, range 2–35 years), 35 MPS IVA patients (average age 15.9 years, range 3.4–56 years), 5 MPS IVB patients (average age 15.8 years, range 12.7–18.3 years), and 144 control subjects (average age 5.1 years, range 0–80 years) are described in Table 1 and Fig. 2a. In the control subjects, levels of mono-sulfated KS were relatively constant (approx. 3.0 µg/ml) up to the age of 10 years, after which they steadily declined before

Table 1 Levels of blood mono- and di-sulfated KS, and proportion of blood di-sulfated KS in total KS of patients with MPS and control subjects

Age	Control	MPS II	MPS IV A	MPS IVB
Mano-sulfated KS (μg/ml)				
0–2.9	3.80 ± 0.82 ($n = 83$)	5.5 ($n = 1$)		
3–4.9	3.91 ± 0.92 ($n = 19$)	6.34 ± 1.42 ($n = 6$)***	4.17 ± 1.39 ($n = 2$)	
5–9.9	3.55 ± 0.59 ($n = 22$)	7.11 ± 2.38 ($n = 23$)***	4.93 ± 0.83 ($n = 10$)***	
10–14.9	2.22 ± 0.53 ($n = 12$)	5.79 ± 2.1 ($n = 13$)***	4.16 ± 1.22 ($n = 9$)***	3.82 ± 0.20 ($n = 2$)
15–19.9	1.80 ± 0.64 ($n = 4$)	4.32 ± 1.46 ($n = 7$)*	3.93 ± 1.07 ($n = 8$)**	2.98 ± 1.67 ($n = 3$)
20-	1.26 ± 0.23 ($n = 4$)	3.47 ± 1.69 ($n = 8$)*	2.04 ± 0.63 ($n = 3$)*	
Di-sulfated (μg/ml)				
0–2.9	1.33 ± 0.34 ($n = 83$)	2.69 ($n = 1$)		
3–4.9	1.56 ± (– = 19)	2.85 ± 1.08 ($n = 6$)***	2.46 ± 1.30 ($n = 2$)	
5–9.9	1.57 ± 0.29 ($n = 22$)	4.05 ± 1.66 ($n = 23$)***	3.08 ± 0.70 ($n = 10$)***	
10–14.9	1.20 ± 0.32 ($n = 12$)	3.47 ± 1.19 ($n = 13$)***	2.99 ± 0.90 ($n = 9$)***	2.32 ± 0.48 ($n = 2$)
15–19.9	1.01 ± 0.31 ($n = 4$)	2.71 ± 0.81 ($n = 7$)**	2.30 ± 0.50 ($n = 8$)***	1.66 ± 1.22 ($n = 3$)
Proportion of blood di-sulfated KS in total KS (%)				
0–2.9	25.9 ± 2.7 ($n = 83$)	32.8 ($n = 1$)		
3–4.9	28.6 ± 3.3 ($n = 19$)	30.4 ± 4.1 ($n = 6$)	36.2 ± 5.0 ($n = 2$)	
5–9.9	30.6 ± 2.3 ($n = 22$)	35.8 ± 3.0 ($n = 23$)***	38.3 ± 4.2 ($n = 10$)***	37.6 ± 3.7 ($n = 2$)
10–14.9	35.2 ± 4.5 ($n = 12$)	37.6 ± 3.6 ($n = 13$)	39.1 ± 2.8 ($n = 9$)*	37.6 ± 3.7 ($n = 2$)
15–19.9	36.1 ± 2.6 ($n = 4$)	39.0 ± 2.5 ($n = 7$)	37.1 ± 4.1 ($n = 8$)	34.4 ± 5.3 ($n = 3$)
20–	42.0 ± 1.1 ($n = 4$)	41.0 ± 3.6 ($n = 8$)	41.6 ± 8.1 ($n = 3$)	

Proportion is calculated as di-sulfated KS/total KS × 100 (%)
Data represent the mean ± SD values
*, ** and ***; significantly different from the control at $p < 0.05$, 0.01, and 0.001, respectively

stabilizing in late teenage years to less than 1.5 μg/ml (Table 1). By contrast, levels of mono-sulfated KS in the blood of patients with MPS IVA were higher than those in age-matched control subjects (Table 1, Fig. 2a). There was not a clear separation of mono-sulfated KS levels between control subjects and patients with MPS IVA (Fig. 2a).

The levels of mono-sulfated KS were also compared between patients with MPS II and IVB and age-matched controls (Table 1, Fig. 2a). In the patients with MPS II, no differences were seen between attenuated and severe phenotypes in mono-sulfated KS. Fifty-four out of 58 (93%) and 43 out of 58 (74%) patients with MPS II had plasma/serum mono-sulfated KS levels more than 2 SD and 3 SD above the mean of age-matched controls, respectively (Table 1). Three out of five (60%) patients with MPS IVB had plasma/serum mono-sulfated KS levels more than 2 SD above the mean of age-matched controls (Table 1).

Blood Di-sulfated KS: Galβ1(6S) → 4GlcNAc(6S)

Plasma/serum di-sulfated KS values in patients with MPS II, IVA, and IVB and the control subjects are shown in Table 1 and Fig. 2b. Levels of di-sulfated KS in the blood from control subjects were also age dependent, similar to that seen for mono-sulfated KS. Di-sulfated KS concentration stayed relatively constant until 15 years of age (approx. 1.5 μg/ml) and then gradually decreased to 0.9 μg/ml in adults (Table 1). The levels of di-sulfated KS in the blood of patients with MPS IVA were significantly higher than those in age-matched control subjects (Table 1, Fig. 2b). Twenty-four out of 35 (69%) and 31 out of 35 (89%) patients with MPS IVA had mono- and di-sulfated KS levels in the blood more than 2 SD above the mean of age-matched controls, respectively. The proportion for di-sulfated KS is significantly higher than for mono-sulfated KS ($p = 0.041$). The likelihood of having di-sulfated KS levels more than 2SD above the mean of age-matched controls is significantly higher than that of having mono-sulfated KS levels in MPS IVA patients, OR = 3.63, $p = 0.048$. Eighteen out of 35 (51%) and 28 out of 35 (80%) patients had mono- and di-sulfated KS levels in the blood more than 3 SD above the mean of age-matched controls. Similarly, the proportion of MPS IVA patients with di-sulfated KS levels more than 3 SD above the mean age-matched control was significantly higher than that with mono-sulfated KS levels, OR = 3.78, $p = 0.01$. These

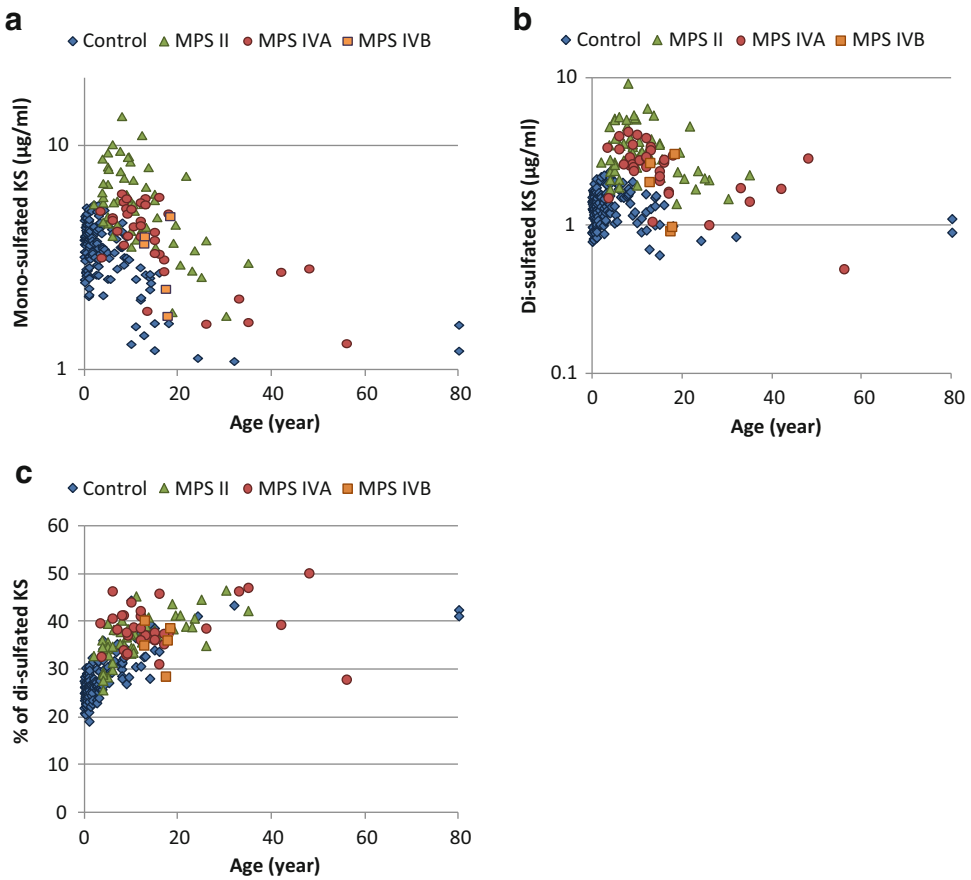

Fig. 2 Levels of blood mono- and di-sulfated KS and proportion of blood di-sulfated KS in total KS of patients with MPS and control subjects. (**a**) Mono-sulfated KS, (**b**) Di-sulfated KS, and (**c**) Proportion of blood di-sulfated KS in total KS. Results of all specimens from patients and control subjects were plotted on a semilogarithmic scale (**a**, **b**) and regular scale (**c**) with respect to age

findings indicate that di-sulfated KS is a better indicator than mono-sulfated KS to distinguish between MPS IVA patients and age-matched controls (cutoff 2 SD, $p = 0.0002$; 3 SD, $p = 0.0003$ by chi-square test). Nevertheless, two MPS IVA patients who had not received any therapy had blood levels of di-sulfated KS that were indistinguishable from controls (Fig. 2b).

Patients with MPS IVB had higher levels of plasma/serum di-sulfated KS than age-matched controls, but differences were not significant for this small set of patients (Table 1, Fig 2b). Fifty-four out of 58 MPS II patients (93%) had di-sulfated KS levels that were more than 2SD above the mean of age-matched controls (Table 1). The enzyme defect in MPS II causes accumulation of heparin sulfate and dermatan sulfate, and consequently elevation of di-sulfated KS must be an indirect effect.

Correlation Between Mono-sulfated KS and Di-sulfated KS Levels in the Blood

A strong correlation between levels of mono- and di-sulfated KS was observed ($r^2 = 0.7733$) for both patients and controls. We found that the contribution of di-sulfated KS to total KS increased with age in the controls but was age independent in MPS IVA patients (Table 1, Fig. 2c). Thus, the proportion of di-sulfated KS in total KS was more discriminating in patients up to 15 years of age. The two patients aged 3.6 and 13.4 who had low levels of di-sulfated KS also had low levels of mono-sulfated KS, so they could be distinguished from controls by the ratio of di-sulfated KS to total KS (Fig. 2c). The fraction of di-sulfated KS in total KS in the patients with MPS II aged 5–10 years significantly increased compared to age-matched controls, but there was considerable overlap in individual values (Table 1, Fig. 2c). Ratio differences between patients with MPS IVB and controls were not significant.

Urine Mono-sulfated KS

The mono-sulfated KS values for the urine samples from 14 MPS IVA patients (average age 16.6 years, range 3.6–56 years), 5 MPS IVB patients (average age 15.8 years, range 12.7–18.3 years), and 44 control subjects (average age 5.1

Table 2 Levels of urine mono- and di-sulfated KS, and proportion of urine di-sulfated KS in total KS of patients with MPS and control subjects

Age	Control	MPS IVA	MPS IVB
Mono-sulfated KS (μg/mg Cre)			
0–29.9	40.36 ± 27.29 ($n = 20$)		
3–9.9	21.56 ± 8.81 ($n = 8$)	97.24 ± 59.45 ($n = 7$)**	
10–19.9	5.71 ± 1.68 ($n = 13$)	45.18 ± 26.80 ($n = 5$)***	25.41 ± 16.33 ($n = 5$)**
20–	1.07 ± 0.33 ($n = 6$)	14.74 ± 2.84 ($n = 2$)	
Di-sulfated KS (μg/mg Cre)			
0–2.9	31.35 ± 14.66 ($n = 20$)		
3–9.9	31.01 ± 18.89 ($n = 8$)	253.06 ± 147.91 ($n = 7$)**	
10–19.9	10.45 ± 2.32 ($n = 13$)	102.18 ± 58.81 ($n = 5$)***	21.02 ± 11.76 ($n = 5$)*
20–	3.59 ± 1.21 ($n = 6$)	27.93 ± 8.45 ($n = 2$)	
Proportion of urine di-sulfated KS in total KS %			
0–2.9	45.38 ± 8.52 ($n = 20$)		
3–9.9	57.20 ± 11.07 ($n = 8$)	71.99 ± 3.45 ($n = 7$)**	
10–19.9	64.92 ± 4.74 ($n = 13$)	69-48 ± 6.16 ($n = 5$)	46.31 ± 2.86 ($n = 5$)***
20–	76.77 ± 1.88 ($n = 6$)	65.12 ± 2.58 ($n = 2$)	

Proportion is calculated as di-sulfated KS/total KS × 100 (%)
Data represent the mean ± SD values
*, ** and ***; significantly different from the control at $p < 0.05$, 0.01, and 0.001, respectively

years, range 0–54 years) are shown in Table 2 and Fig. 3a. Levels of mono-sulfated KS in the urine were also age dependent (Fig. 3a). The level was highest in newborns (40 μg/mg Cre) and decreased until 15 years of age and reached a plateau in twenties and older (1.0 μg/mg Cre, Table 2). The levels of mono-sulfated KS in the urine of patients with MPS IVA were significantly higher than those in age-matched control subjects (Table 2, Fig. 3a). All patients with MPS IVA had levels of mono-sulfated KS that were higher than 3 SD above the mean of the age-matched controls. In contrast to blood samples, levels of urine mono-sulfated KS in patients with MPS IVB significantly increased as well, and all of them were more than 3 SD above the mean of age-matched controls (Table 1, Fig. 3a).

Urine Di-sulfated KS

In the control subjects, levels of urine di-sulfated KS were relatively constant (approx. 30 μg/mg Cre) up to 10 years of age and, thereafter, steadily declined to less than 5.0 μg/mg Cre in late teenage years (Table 2). The levels of urine di-sulfated KS in patients with MPS IVA were significantly higher than those in age-matched control subjects (Table 2, Fig. 3b). All patients with MPS IVA had urine levels of di-sulfated KS that were more than 3 SD above the mean of the age-matched controls. Levels of urine di-sulfated KS in patients with MPS IVB significantly increased, and two out of five patients with MPS IVB were more than 3 SD above the mean of age-matched controls (Table 2, Fig. 3b).

Correlation Between Mono-sulfated KS and Di-sulfated KS Levels in the Urine

There was a strong correlation between mono- and di-sulfated KS for both patients and controls ($r^2 = 0.7556$). We found that the contribution of di-sulfated KS to total KS in the urine was higher than that in the blood and also increased with age in the controls and was age independent in MPS IVA patients (Fig. 3c, Table 2). The proportion of di-sulfated KS in total KS in the patients with MPS IVB was significantly lower than that in patients with MPS IVA ($p < 0.001$) and age-matched control subjects ($p < 0.001$) (Fig. 3C, Table 2).

Discussion

In this study, we have demonstrated (1) that the peak of di-sulfated KS is separated from the mono-sulfated KS as a pure single peak by the LC column using the *m/z* 542 di-sulfated precursor ion and *m/z* 462 product ion, (2) that the levels of blood and urine mono-sulfated and di-sulfated KS in control subjects are age dependent and decline with age, (3) that blood and urine mono- and di-sulfated KS levels in patients with MPS IVA are significantly higher than those

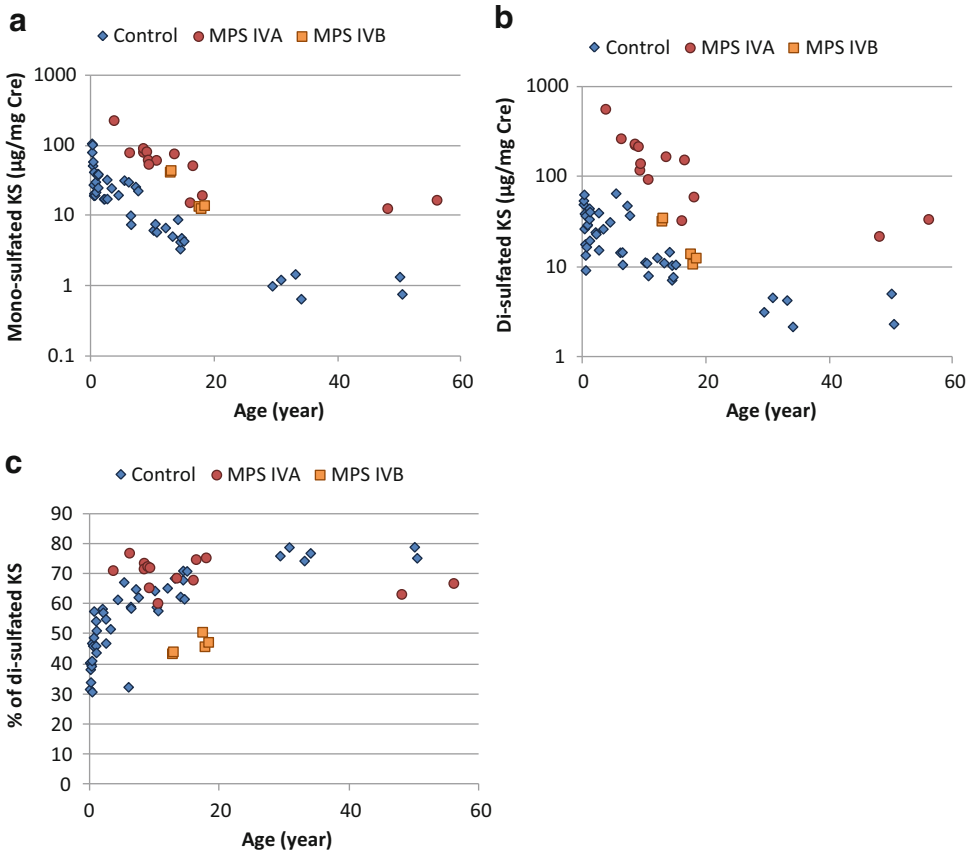

Fig. 3 Levels of urine mono- and di-sulfated KS and proportion of blood di-sulfated KS in total KS of patients with MPS and control subjects. (**a**) Mono-sulfated KS, (**b**) Di-sulfated KS, and (**c**) Proportion of urine di-sulfated KS in total KS. Results of all specimens from patients and control subjects were plotted on a semilogarithmic scale (**a**, **b**) and regular scale (**c**) with respect to age

in age-matched control subjects, (4) that the level of blood and urine di-sulfated KS distinguishes control subjects and patients with MPS IVA more clearly than that of mono-sulfated KS, (5) that the ratio of blood di-sulfated KS to total KS increases with age and was significantly elevated in patients with MPS IVA under 15 years of age compared to control subjects, (6) that levels of mono- and di-sulfated KS in the urine provide a clear difference between patients with MPS IVA and MPS IVB and age-matched controls, and (7) that blood mono- and di-sulfated KS levels are significantly higher in patients with MPS II than those in age-matched control subjects. In addition, total KS and levels of sulfation depend on species and tissues examined.

KS is comprised of repeating sulfated disaccharide units of Gal and GlcNAc (Galβ1-4GlcNAcβ1-3). The process of KS biosynthesis consists of (1) *N*-acetylglucosaminylation, (2) 6-sulfation of a GlcNAc residue, and (3) galactosylation. KS polymers are extended by the action of glycosyltransferases that alternately attach Gal and GlcNAc residues. KS is generally sulfated on C (6) of GlcNAc, while the C (6) of Gal is sulfated to a variable extent, depending on the tissue and age (Bhavanandan and Meyer 1968).

In previous studies, we identified mono- and di-sulfated KS using the same MRM condition of *m/z* 462 precursor ion and *m/z* 97 product ion using LC-MS/MS, but the two forms were not separately quantified completely (Oguma et al. 2007a, b; Hintze et al. 2011), resulting in an overlap of the values between mono- and di-sulfated KS. In this study we detected and quantified the di-sulfated KS using the *m/z* 542 precursor ion and *m/z* 462 product ion which is specific to di-sulfated KS and confirmed that the peak of di-sulfated KS is separated from the mono-sulfated KS as a pure single peak by the LC column. Thus, we have established more appropriate conditions for detecting mono- and di-sulfated KS separately compared to the previous method (Oguma et al. 2007a). We clearly show that KS in shark cartilage is mostly di-sulfated, while in bovine cornea mono-sulfated KS is dominant. This finding is consistent with the fact that KS chains of fibromodulin in cartilage are highly sulfated compared to that in the cornea (Lauder et al. 1997; Nieduszynski et al. 1990). Our data also show that the level of both mono- and di-sulfated KS in the blood of control subjects decline with age, while the degree of sulfation (i.e., proportion of di-sulfated KS in

total KS) increases with age. Both mono- and di-sulfated forms of KS are found in the growth plates, articular cartilage, ECM, cornea, and brain. This age-dependent alteration of KS level in the plasma/serum can be explained by decreased KS synthesis after the growth plate is closed.

An increase of sulfation in blood and urine KS is compatible with that observed in the cartilage and cornea during normal aging (Liles et al. 2010; Brown et al. 1998). Gal sulfation levels increase during adolescence and early adulthood and then remain fairly constant in human articular cartilage (Brown et al. 1998).

KS is involved in specific biological functions including tissue hydration, cellular recognition of protein ligands, axonal guidance, cell motility, and embryo implantation (Chakravarti et al. 1998; Weyers et al. 2013; Imagama et al. 2011; Funderburgh et al. 1997; Graham et al. 1994). KS has been implicated in physiological and pathological status: (1) cushion in joints, (2) glial scar formation in spinal cord injury, (3) transparency in the cornea, and (4) skeletal dysplasia in patients with MPS IV. However, the physiological roles and distributions of the individual sulfated KS and its increased sulfation with age are not fully understood due to lack of accurate quantitative method to measure mono- and di-sulfated KS. The current method will shed light on the implication of physiological roles and distributions of the individual sulfated KS.

Total KS level in the plasma/serum can be used as a biomarker for MPS IVA (Tomatsu et al. 2008, 2010c, 2012b, 2013a; Hintze et al. 2011; Martell et al. 2011); however, there are substantial overlaps of KS values between control subjects and MPS IVA patients, especially for patients older than 15 years of age, and consequently total KS alone is not a good biomarker for MPS IVA patients of all ages. GALNS, an enzyme involved in the first step of degradation of polymer KS, hydrolyzes the C (6) sulfate of Gal from di-sulfated KS, Gal(6S)β1 → 4GlcNAc(6S), to produce mono-sulfated KS, Galβ1 → 4GlcNAc(6S). Therefore, as GALNS is deficient in patients with MPS IVA, di-sulfated KS would be expected to increase in the plasma/serum more clearly than mono-sulfated KS, as confirmed in this study. In this study, di-sulfated KS was found to improve discrimination between MPS IVA patients and controls compared to total KS and mono-sulfated KS and consequently may be a better biomarker for early diagnosis, screening, assessment of disease severity, and monitoring therapeutic efficacy for MPS IVA.

Levels of di-sulfated KS do not distinguish all MPS IVA patients from controls and may be affected by disease status. Most KS is synthesized in cartilage, and consequently blood KS reflects the amount of KS derived from cartilage. If cartilage turnover is reduced by prior loss, reduced activity, or other means, total blood KS could be lower in MPS IVA patients than in controls. Reduced total turnover of KS can be controlled in part by measuring the ratio of di-sulfated KS in total KS. This ratio allowed us to distinguish the two MPS IVA patients under 20 years of age who had low levels of di-sulfated KS from controls. Overall, this study has shown that the level of di-sulfated KS distinguishes patients with MPS IVA and control subjects better than that of mono-sulfated KS alone.

It is noteworthy that levels of KS are elevated in the blood from not only MPS IVA patients but around 90% of MPS II and other types of MPS patients (Tomatsu et al. 2005; Rowan et al. 2013; current study). The level of KS in the blood from MPS II patients was as high as that seen in MPS IVA patients. MPS II is caused by deficiency of iduronate-2-sulfatase, an enzyme that is not directly involved in the degradation of polymer KS, and consequently the mechanism of elevation of KS in these patients must differ from that seen in MPS IVA patients. In contrast to results from the blood, only 35% of levels of KS in the urine from MPS II patients were more than 2SD above the mean of age-matched controls. Several mechanisms have been proposed to account for a secondary elevation of blood KS in patients with MPS II and other types of MPS (Tomatsu et al. 2005): (1) Synthesis of KS could be induced by inflammation caused by storage of other GAGs. (2) GALNS activity could be inhibited by the increased concentrations of heparan sulfate in patients with MPS II (and some other MPS types) (Rowan et al. 2013). (3) KS secretion into the circulation could be due to damage of cartilage and its ECM caused by accumulation of other GAGs and its subsequent inflammation. It is well known that the degradation of proteoglycans occurs early in joint damage and that fragments are released into the synovial fluid and subsequently the serum (Dingle et al. 1975; Ratcliffe et al. 1988). Blood KS levels have been associated with severity of skeletal dysplasia in mouse models of MPS I, III, IVA, and VII (Rowan et al. 2013). (4) Polymer KS could be co-deposited with the other accumulated GAGs, hindering the interaction between KS and its catabolizing enzymes. (5) Alterations in the extent and distribution of fucosylation, sialylation, and sulfation on KS could make KS resistant to degradation (Tai et al. 1994). One or a combination of these mechanisms could contribute to a secondary elevation of KS. Regardless of the mechanism, our data show that KS levels in the blood is a good biomarker for MPS II and is better at distinguishing MPS II patients from controls than KS levels in the urine. A larger study including more patients with MPS II and other types of MPS will be required to establish age-dependent changes of KS in these disorders.

We did not see a significant elevation of either sulfated form of KS in the plasma/serum of patients with MPS IVB. Patients with MPS IVB have a milder phenotype compared

with those in MPS IVA, so lower levels of secretion of KS would be expected. The proportion of di-sulfated KS in patients with MPS IVB is not as high as that seen in MPS IVA. There were only 5 MPS IVB patients in this study, and none have severe disease and none were younger than 12 years of age. Further studies including younger and severe-type patients with MPS IVB are required to determine the significance of sulfation levels of KS for this disorder.

Urine mono- and di-sulfated KS showed an age-dependent decline, similar to that seen for sulfated KS in the plasma/serum. Elevation of total urine KS in patients with MPS IVA has been reported (Tomatsu et al. 2010c; Martell et al. 2011). KS in the urine more clearly distinguishes patients with MPS IVA and MPS IVB from age-matched controls than levels in the plasma/serum, even for older patients. All urine samples from the five MPS IVB patients had a ratio of di-sulfated KS to total KS that is lower than that in age-matched controls. While deficiency of GALNS in MPS IVA would be expected to increase the proportion of di-sulfated KS, deficiency of β-gal in MPS IVB would not be expected to have any direct effect on sulfation levels. A larger study with more control subjects and patients is required to establish di-sulfated KS in the urine as a biomarker for MPS IVA and MPS IVB and to determine the mechanism by which sulfation levels are affected differentially in the plasma/serum and urine of MPS IVA and IVB patients.

Nevertheless, the proportion of di-sulfated KS in total KS in urine appears to be a useful biomarker to distinguish patients with MPS IVA and MPS IVB. Although not tested in this study, the measurement of mono- and di-sulfated KS could be potentially useful for monitoring the outcome of ERT and other therapies for MPS IVA.

Conclusions

In conclusion, we have developed a method to evaluate mono- and di-sulfated KS levels in a variety of specimens by LC-MS/MS systems, leading to understanding of species-specific and/or tissue-specific KS level and its sulfation level, and the age-dependent alteration of KS and its sulfation level. Significant difference in sulfation levels of KS between control subjects and patients with MPS IVA demonstrates that the di-sulfated KS is a potential biomarker for this disease.

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Compliance With Ethical Guidelines

Conflict of Interest

All the authors have contributed to this "Original Article" and have no conflict of interest with any other party.

Tsutomu Shimada, Eriko Yasuda, Robert W. Mason, Jobayer Hossain, William G. Mackenzie, Yuniko Shibata, Seiji Yamaguchi, Yasuyuki Suzuki, Kenji E. Orii, Toshiyuki Fukao, Tadao Orii, Adriana M. Montaño, Francyne Kubaski, Roberto Giugliani, and Shunji Tomatsu declare that they have no conflict of interests.

Informed Consent

The samples were obtained with the informed consent according to IRB approval at each institute.

Animal Rights

Not applicable.

Contributions to the Project

Tsutomu Shimada: He has contributed to the concept of the project, planning, performance of experiments (LC-MS/MS), data analysis, and reporting of the work described in the article.

Shunji Tomatsu: He is a Principal Investigator and is responsible for the entire project. He has contributed to the concept of the project, planning, analysis of data, and reporting of the work described in the article. He organized and communicated the entire team for this project

Robert W. Mason: He has contributed to the planning, performance of LC-MS/MS, data analysis, and reporting of the work described in the article.

Eriko Yasuda: She has contributed to collecting samples, data analysis, and reporting of the work described in the article.

Jobayer Hossain: He contributed to data analysis and statistics and reporting of the work described in the article.

William G. Mackenzie: He has contributed to collecting samples, data analysis, and reporting of the work described in the article.

Yuniko Shibata: She has contributed to data analysis and reporting of the work described in the article.

Adriana M. Montaño: She has contributed to data analysis and reporting of the work described in the article.

Francyne Kubaski: She has contributed to collecting samples, data analysis, and reporting of the work described in the article.

Roberto Giugliani: He has contributed to collecting samples, data analysis, and reporting of the work described in the article.

Seiji Yamaguchi: He has contributed to collecting samples, data analysis, and reporting of the work described in the article. He and his team at Shimane University worked with Dr. Tomatsu.

Yasuyuki Suzuki: He has contributed to collecting samples, data analysis, and reporting of the work described in the article.

Kenji E. Orii: He has contributed to collecting samples, data analysis, and reporting of the work described in the article.

Toshiyuki Fukao: He has contributed to collecting samples, data analysis, and reporting of the work described in the article. He and his team at Gifu University worked with Dr. Tomatsu.

Tadao Orii: He has contributed to collecting samples, data analysis, and reporting of the work described in the article.

Highlights

- Sulfation level of KS varies with age and species.
- Importance of level of di-sulfated KS and ratio of KS sulfation.
- Di-sulfated KS can be used as a biomarker for several types of MPS.
- Level of blood KS is age dependent and species dependent.
- The reader will understand the importance of a new biomarker for MPS II, IVA, and IVB.

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RESEARCH REPORT

Metabolic Clinic Atlas: Organization of Care for Children with Inherited Metabolic Disease in Canada

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Abstract *Introduction*: Nearly all children in Canada with an inherited metabolic disease (IMD) are treated at one of the country's Hereditary Metabolic Disease Treatment Centres. We sought to understand the system of care for paediatric IMD patients in Canada in order to identify sources of variation and inform future research priorities.

Methods: Treatment centres were contacted by email and invited to complete a web-based survey. The questionnaire addressed, for each centre, the population size served and scope of practice, available human resources and clinic services and research capacity. Survey responses were analyzed descriptively.

Results: We received responses from 13 of the 14 treatment centres invited to participate. These centres represent at least 85% of the Canadian population, with over half of the centres located in southern Ontario and Quebec. All centres reported paediatric patients with IMDs as their main patient population. A variety of dedicated staff was identified; every centre reported having at least one physician and one dietician. The most common ancillary services available included telehealth (11/12 respondents) and biochemical genetic laboratory testing (10/12), with a high variability of access to on-site laboratory tests. A majority of centres indicated access to additional off-site services, but barriers to these were reported. All but one centre indicated previous experience with research.

Conclusions: The variation we identified in the organization of care highlights the need to investigate the association between practice differences and health outcomes for paediatric IMD patients to inform policies that establish equitable access to services that are beneficial.

Introduction

Although individually rare, collectively, inherited metabolic diseases (IMDs) represent a substantial population health burden in Canada and internationally. Studies have estimated the Canadian birth prevalence for diagnosed IMDs to be from 1 in 2,500 to 1 in 1,900 (Applegarth et al. 2000; Auray-Blais et al. 2007). The currently observed Canada-wide prevalence of all IMDs is likely somewhat higher than both these estimates, due to a number of factors including improved identification strategies such as expanded newborn screening (Schulze et al. 2003; Wilcken et al. 2003), improved diagnostic services and awareness of less recognized conditions, longer survival for IMD patients due to newly developed therapies and immigration of populations at higher risk of particular IMDs.

The majority of health care in Canada is publically funded to provide care to all based on need rather than ability to pay (Health Canada 2011). Public health insurance programmes in all provinces and territories cover both primary and secondary physician and hospital care, with limited coverage for allied health services outside of hospitals (Health Canada 2011). Coverage for pharmaceuticals and other products relevant to IMD, such as medical foods or supplements, varies amongst provinces and territories (Health Canada 2011). Nearly all children in Canada diagnosed with an IMD receive specialized care at one of 16 Hereditary Metabolic Disease Treatment Centres. At these centres, paediatric IMD patients have access to specialist physicians and services to manage diagnosis, treatment and follow-up care.

The goal of this study was to provide a broad overview of the organization of specialized care for IMD children in Canada, in order to identify areas of practice variation and inform priorities for future research examining how service provision affects outcomes. We invited Canadian Hereditary Metabolic Disease Treatment Centres to complete a survey to:

1. Describe their centre's scope of practice, in terms of the population and types of patients served
2. Identify the human resources available and the specific clinical services offered or to which they have access
3. Describe their research capacity

Methods

Sample Selection and Survey Implementation

The recently established Canadian Inherited Metabolic Diseases Research Network (CIMDRN) is a practice-based research network that aims to inform care and ultimately to improve outcomes for children with IMD in Canada and beyond (Potter et al. 2013). Fourteen of the 16 Canadian Hereditary Metabolic Treatment Centres form the practices in this research network. By email, we invited one metabolic physician at each of these 14 centres to participate in the survey. Physicians were asked to seek assistance from other knowledgeable staff at the centre as needed. The survey was administered online, between January and March 2013 using FluidSurveys (www.fluidsurveys.com), a secure online questionnaire tool. Ethics approval was obtained from The Children's Hospital of Eastern Ontario Research Ethics Board.

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Questionnaire Content and Data Analysis

Survey questions were divided into three categories:

1. Scope of practice (patient populations)
2. Human resources and clinic services
3. Research capacity

The questionnaire required approximately 30 minutes to complete. We used Microsoft Excel (2011) to conduct descriptive analysis, reporting relevant means and proportions.

Results

Scope of Practice

Of the 14 centres invited to participate in the survey, we received completed surveys from 13 (response rate of 93%). These 13 centres care for the majority of IMD children in all ten provinces and three territories in Canada, serving geographic catchments with populations of 500,000 to over six million and representing at least 85% of the Canadian population (Fig. 1). Over half of the participating centres are located in southern Ontario and Quebec (Fig. 1). Five centres were classified as large clinics serving populations of over two million, four centres were considered mid-sized serving >1–2 million people and the remaining four were classified as small serving one million or fewer people.

All centres reported their main patient population as IMD patients; phenylketonuria (PKU) was the most common diagnosis. Some clinics also reported providing services to patients with non-IMD conditions (e.g. neurologic disorders, autism, other genetic diseases). All centres reported at least 20 paediatric IMD patients currently in their care; six centres (46%) reported having over 100 paediatric IMD patients. Eight respondents indicated a second clinic in their catchment area providing some diagnostic or treatment services to IMD patients; these were mainly neurology clinics or adult care facilities and a specific clinic in New Brunswick for PKU patients. Four (31%) centres reported a separate adult clinic in the same catchment area to help manage the transition from paediatric to adult care; 12/13 centres reported having adult (>18 years old) IMD patients under their care, including pregnant women.

Human Resources and Clinic Services

All participating centres reported at least one physician and one registered dietician on staff. Other staff identified included nurses, genetic counsellors, pharmacists, psychologists, social workers, administrative assistants and research coordinators (Fig. 2a). Number of staff varied across centres (Fig. 2b), and as expected, larger centres reported more staffing resources. Roles for staff members included clinical, administrative, teaching and research responsibilities. Physicians, nurses, dieticians and genetic counsellors are most heavily involved in primary patient contact, initial consultations and case coordination, with some contributions from social workers and psychologists. 77% (10/13) of centres reported that they use an inter-professional model of care for their patients. We defined 'inter-professional care' in the questionnaire as an integrated approach to health-care delivery in which the collaboration amongst practitioners of different disciplines or with different skills and knowledge allows for the delivery of patient health care by the most appropriate health-care practitioner.

The most common ancillary services provided by the centres (i.e. in addition to direct patient care) were telehealth (11/12 centres who responded to these questions), biochemical genetic laboratory testing (10/12), a specialized pharmacy (8/12) and a dispensary for medical foods/supplements (8/12). Patient/family workshops (7/12) and patient/family support groups (6/12) were also provided by some centres. Over 80% of the centres reported access to other services for patients and families, either within or outside the clinic. These services included additional prenatal genetic diagnostic care (11/13 centres), genetic counselling (10/12) and social work (11/13). When asked about barriers to patients' access to services, several centres reported challenges associated with access to nutritional services (e.g. feeding devices, parenteral nutrition) and psychological services, mainly due to long wait lists and/or being located off-site. There was high variability amongst the 12 centres that reported on the availability of specific on-site laboratory tests (Table 1). The tests most commonly available were urinalyses for organic acids and plasma/urine amino acid analyses (92% of centres).

Research Capacity

Twelve of the 13 responding centres (92%) indicated previous involvement with research including industry-funded trials (11/12 centres), survey or interview-based studies (9/12), diagnostic studies (8/12), retrospective studies using chart abstraction (7/12) and nonindustry-funded trials (5/12). Staff responsibilities specific to research were highly variable, but involvement included physicians, nurses, dieticians, genetic counsellors, administrative assistants and research coordinators. Important reasons for participating in research included: contributing to the improvement of care for IMD patients (100%), contributing to the scientific understanding of

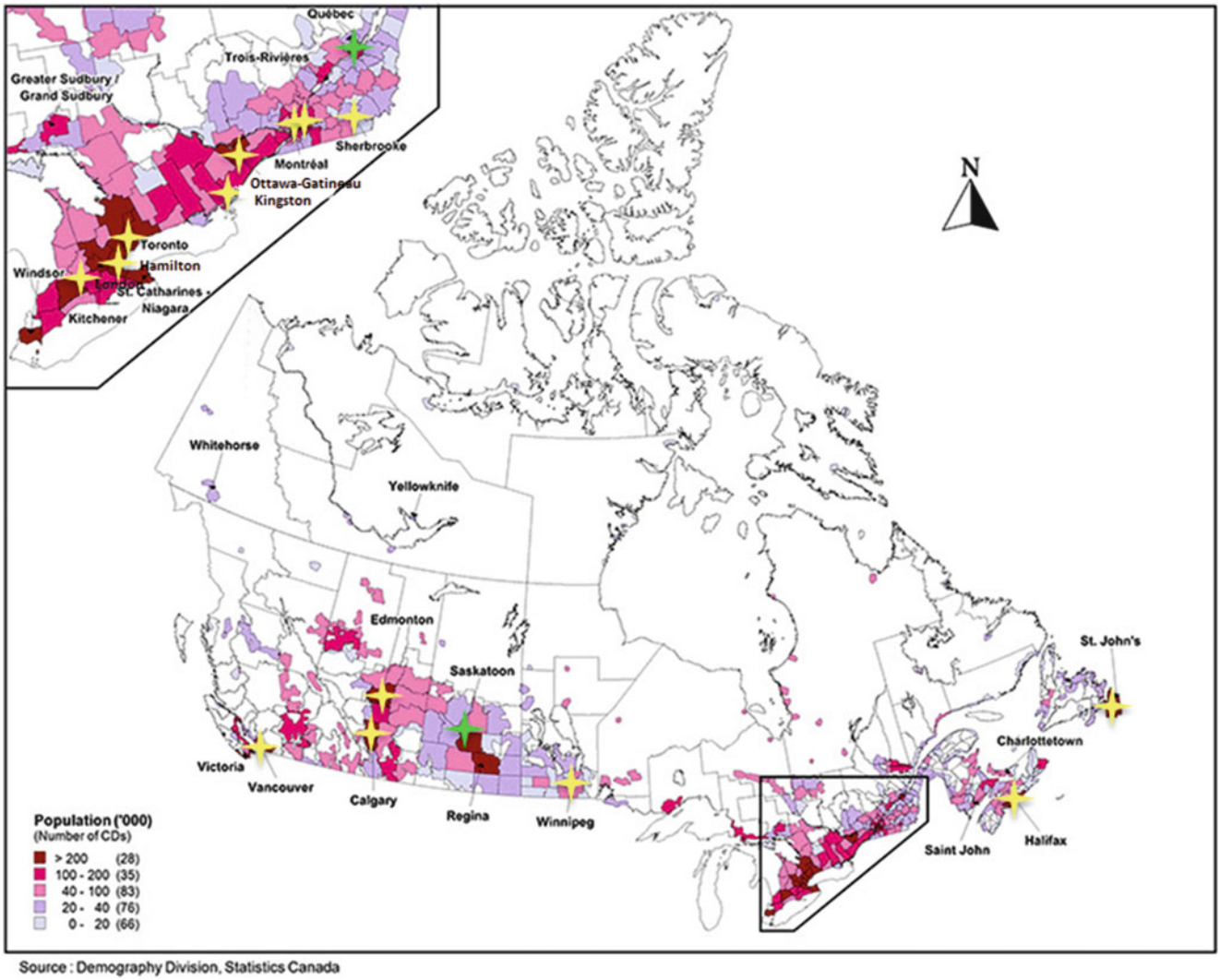


Fig. 1 Population density map of Canada using the most recent census data (Statistics Canada 2011) indicating the location of all 16 Hereditary Metabolic Disease Treatment Centres. Centres participating in CIMDRN are indicated in yellow. Centres highlighted in green were not part of CIMDRN at the time of data collection. Survey data was collected from January–March 2013

IMDs (100%) and building professional and inter-centre relationships (92%). Centres expressed barriers to involvement in research related to workload (100%) and concerns about research sustainability due to limited funds (85%).

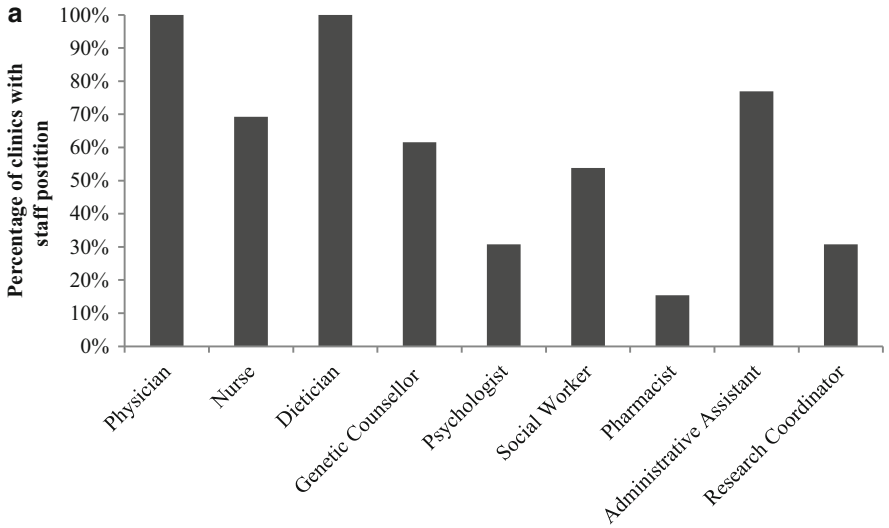
Discussion

Summary and Interpretation

We found important variation in the organization of care in Canada for paediatric IMD patients. Variation in IMD management has been noted in other jurisdictions (Leonard 2006), and although the results of our survey are from Canadian clinics, there is much that could be pertinent to health care for IMDs internationally. Specifically, the variation we identified highlights the need for evaluative evidence to better understand whether these differences in care are associated with differences in patient outcomes and provides an opportunity to generate that evidence using observational study designs that capitalize on practice variation as 'natural experiments' (Horn and Gassaway 2007).

Our survey results demonstrate variation in human resources and services available at Canadian IMD treatment centres. This variation in clinical infrastructure may reflect clinical heterogeneity in the complex needs of patients with different types of IMDs, including the needs of specific high-risk populations at some of the centres (e.g. First Nations or founder populations, immigrant communities). Further research is needed to determine a more specific

a



b

Staff Position	Full-time Equivalent (FTE) Staff		
	Median	Minimum	Maximum
Physician	1	0.3	4.5
Nurse	0.5	0	3
Dietician	1	0.2	2.8
Genetic Counsellor	0.1	0	2
Psychologist	0	0	0.5
Social Worker	0.1	0	1
Pharmacist	0	0	0.2
Administrative Assistant	0.6	0	3
Research Coordinator	0	0	3

Fig. 2 (**a**) Proportion of IMD centres ($n = 13$) reporting staff position at their centre and (**b**) median number of full-time equivalent staff for participating IMD centres ($n = 13$), indicating minimum and maximum values

profile of IMD patients across centres; precise patient numbers for these rare diagnoses are challenging to estimate accurately, as is the distribution of patients by age, ethnic background, disease severity and presence of co-morbidities. Alternatively, differences may reflect differences in provincial/territorial health service organization and prioritization.

Similar to trends in both the United States and the United Kingdom, we also found that many adult IMD patients in Canada are being treated in paediatric centres (Burton et al. 2006; Berry et al. 2013). Consideration of the transition from paediatric to adult health care is a current priority as new therapies make it possible for greater numbers of IMD patients to survive into adulthood (Dionisi-Vici et al. 2002; Lee 2002; Mütze et al. 2011). There is growing evidence that suggests it is beneficial for centres to establish transition protocols and, depending on the volume of patients in a centre, separate clinics for adult patients (Lee 2002; Mütze et al. 2011; Sirrs et al. 2014).

Not surprisingly, centres that serve a larger population have more resources. However, what is not known is the ratio of resources to patients at the centres, whether the organization of resources at the centres is effective or not and how these factors are linked to clinical and patient-centred outcomes. There is also limited evidence regarding the value of allied health services for particular IMD populations, such as psychology and occupational therapy. Similar to our study findings, barriers to these services have been reported elsewhere (Camfield et al. 2004; Berry et al. 2013). Research to answer these questions is necessary to determine what resources and services should be offered at each centre to optimize health outcomes.

Table 1 Summary of laboratory tests available on-site at participating IMD centres ($n = 12$)

Test provided	% 'Yes'
Organic acid analysis	92
Plasma/urine amino acids	92
Total and free carnitine	67
Bloodspot phenylalanine	67
Bloodspot acylcarnitine profile	58
Plasma acylcarnitine profile	50
Lysosomal enzymology	50
Urine MPS fractionation	42
Urine oligosaccharide fractionation	42
Respiratory chain enzymology	33
mtDNA point mutation analysis	33
mtDNA quantification and deletion/duplication analysis	25
Other analytes (e.g. purines, succinylacetone, VLCFA, etc.)	67
Other enzymology (e.g. VLCAD, Gal-1-PUT, etc.)	42
Other molecular analyses for IMD	33
Other bloodspot tests	33

Note: Data were missing on this survey question for one of the participating centres

In addition to availability, access is another concern. Although the location of the treatment centres is proportionate to the geographic distribution of the Canadian population, as well as the birth prevalence of IMDs, patients living in more northern or remote areas have to travel a considerable distance for specialized metabolic care. Further research is needed to determine ascertainment of IMD patients in remote areas and how clinical and patient-centred outcomes are affected by having limited geographic access to services. There were several centres with only one dedicated IMD physician. This may pose problems with access to care for IMD patients outside regular hours. Although our survey was not able to delve into this issue in detail, it is an important consideration for further study. Services such as laboratory testing can be accessed elsewhere; however, turnaround time, costs and quality assurance are important to consider. All centres must send out samples for some metabolic and/or genetic testing as not even the largest centres are able to perform all relevant testing, thus adding another source of variation amongst centres which could potentially impact on care delivery. This issue also raises the question of whether some services can be effectively delivered in patients' home communities rather than at a distant metabolic clinic.

Although Canada has established centres to provide care for IMD patients, the variation in clinical infrastructures we identified reflects the lack of a central mechanism to guide minimum care standards. This is further exemplified with the lack of a national strategy for newborn screening. In Canada, newborn screening programmes are unique to each province and territory, differing in the panel of disorders screened, technologies used, follow-up processes, legal structures and governance (Therrel and Adams 2007; Wilson et al. 2010; Morrison and Dowler 2011). Canada also lacks a national metabolic laboratory network that could set standards and provide coordination for biochemical genetic laboratory tests, on which IMD patients rely for diagnosis, monitoring and informing treatment (Burton et al. 2006; Leonard 2006; Leonard and Morris 2006). Reimbursement decisions for drug funding also differ across the provinces and territories. Health Canada recently announced the development of a Canadian orphan drug framework intended to provide Canadians with better, timelier access to orphan drugs and to encourage and facilitate clinical research in the area of rare diseases (Lee and Wong 2014). Once practice-based research studies have determined what aspects of the system of care have the greatest impact on health outcomes for IMD patients, a mechanism to implement research findings nationally will be critical.

In conclusion, although the majority of Canadian paediatric IMD patients are receiving care from one of the Hereditary Metabolic Disease Treatment Centres, the specific resources and services available vary greatly across the country. This variation in the organization of care for IMDs across Canada presents a unique opportunity for observational practice-based research to determine whether patterns of care are associated with clinically significant differences in patient outcomes.

Limitations

Despite a high response rate (93%), our study was limited by the small number of responding centres ($n = 13$), because of the small number of IMD treatment centres in Canada. Nevertheless, the geographic catchments served by the responding centres support the representativeness of our sample. The three 'missing' centres would likely add further variation to that found amongst the surveyed centres. Survey questions were broad to account for variability amongst clinics; however, some important details may have been missed that could help explain some of the observed variation in services.

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Concise 1 Sentence Take-Home Message (Synopsis) of the Article, Outlining What the Reader Learns from the Article

Variation in the organization of care for paediatric IMD patients in Canada identifies a need to investigate the association between practice differences and health outcomes to enable policy development that will ensure access to services that are effective, equitable and affordable.

Compliance with Ethics Guidelines

Conflict of Interest

Monica F Lamoureux, Kylie Tingley, Jonathan B Kronick, Beth K Potter, Alicia KJ Chan, Doug Coyle, Linda Dodds, Jane Gillis, Grant Mitchell, Anne-Marie Laberge, Julian Little, Kathy N Speechley, Sylvia Stockler, Yannis Trakadis, Lesley Turner, Kumanan Wilson and Pranesh Chakraborty declare that they have no conflict of interests.

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Sarah Dyack has received honoraria, research and/or travel funds from Shire and/or Genzyme Corporation.

Annette Feigenbaum has received funding for industry-sponsored research and honoraria from BioMarin Pharmaceutical and Hyperion Therapeutics on studies unrelated to the present study, as well as honoraria from Symbiotix and Medaccess for educational programmes.

Michael Geraghty has funding from BioMarin for a clinical trial as a site-PI.

Cheryl Rockman-Greenberg received research grant support, but no personal financial compensation, for clinical trials from BioMarin, Genzyme Canada, Shire Human Genetic Therapies (Canada) Inc. and Alexion Pharmaceuticals. She also received honoraria from Alexion for industry-sponsored lectures, symposia and webinars and travel reimbursement and consultation fees from the Actelion National Advisory Board for Niemann-Pick C disease.

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Chitra Prasad is the local site principal investigator for PKU 016 trial and the local site investigator for the Replegal trial.

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Clara Van Karnebeek is a Co-I on a Shire-funded study and a Co-PI on a Ultragenyx-funded study.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Completion and submission of the survey constituted consent to participate in this study. This article does not contain any studies with animal subjects performed by any of the authors.

Contributions

MFL, BKP. Concept and design, analysis and interpretation of data, drafting article and critical revisions of article

KT. Analysis and interpretation of data, drafting article and critical revisions of article

PC, JBK. Concept and design, analysis and interpretation of data and critical revisions of article

AKJC, DC, LD, SD, AF, MG, JG, CRG, AK, JL, BM, JM, AM, JJM, GM, AML, MP, CP, KS, KNS, SS, YT, LT, CVK, KW. Concept and design, and critical revisions of article

PC. Guarantor

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RESEARCH REPORT

Maternal Phenylketonuria: Long-term Outcomes in Offspring and Post-pregnancy Maternal Characteristics

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Abstract Maternal phenylketonuria (MPKU) is a well-recognized complication of PKU and one of the most potent teratogenic syndromes of pregnancy. Virtually all offspring from untreated pregnancies in women with classic PKU have intellectual disabilities and microcephaly. Congenital heart disease and intrauterine growth retardation occur many times more often than expected in the general population. Control of maternal blood phenylalanine during pregnancy prevents most if not all of these complications. Previous studies demonstrated the benefits of treatment in terms of birth parameters and early development. In this study, physical examinations, a medical history, and neuropsychological evaluation were obtained in 47 children from 24 mothers with PKU who received treatment during pregnancy. Mothers were interviewed and administered an abbreviated IQ test. Associations between maternal factors and offspring outcomes were also analyzed.

The 21 male and 26 female offspring ranged in age from 1 month to 26 years with 21 (62%) over 6 years. Results indicated mean intercanthal distances above the 70th percentile. Microcephaly was present in 19% of offspring, with head circumference below the third percentile. None of the offspring had cardiac anomalies. Mean offspring IQ was 94 ± 19, with 12% performing in the range of intellectual disability (IQ < 70). Among children >5 years of age, 25% had learning disabilities, 31% had attention deficit hyperactivity disorder (ADHD), 22% were on ADHD medication, and 34% had a diagnosis of anxiety and/or depression. Among the 24 mothers, 12 reported following the diet for PKU. Only one woman on diet had a blood phenylalanine concentration <360 μmol/L (recommended range) and the majority had indications of poor nutritional status. Mean maternal Full Scale IQ was 94 ± 16 (range = 61–117), with 25% performing in the borderline intellectual range (IQ < 85). Verbal IQ was significantly lower than Performance IQ ($p = 0.01$, CI 2.7, 16.1). On the self-report Beck Depression Inventory, Second Edition, 25% received scores indicating mild to moderate depression, and on the Beck Anxiety Inventory, 46% reported mild to moderate anxiety. Offspring IQ correlated with maternal metabolic control during pregnancy ($r = 0.51$), maternal IQ ($r = -0.62$), and socioeconomic position ($r = -0.48$). Offspring with ADHD, learning disabilities, or emotional disturbances were more likely to have mothers with anxiety and/or depression. To ensure optimal offspring outcomes, healthcare providers need to assess maternal nutrition, blood phenylalanine concentrations, cognitive abilities, and socioeconomic position. Interventions can then be initiated that reduce psychosocial stressors and enhance adherence to diet and positive parenting, which in turn can lead to better cognitive functioning, behavior, and emotional well-being in their children.

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Introduction

Maternal phenylketonuria (MPKU) is a well-recognized complication of PKU and one of the most potent teratogenic syndromes of pregnancy. Virtually all offspring from untreated pregnancies in women with severe PKU have intellectual disabilities and microcephaly. Congenital heart disease and intrauterine growth retardation occur many times more often than expected in the general population (Lenke and Levy 1980). The frequencies of these abnormalities in offspring are lower when the woman has a milder form of PKU but are still much greater than normally expected (Lenke and Levy 1980; Levy et al. 2003; Güttler et al. 2003).

Control of the maternal blood phenylalanine during pregnancy prevents most if not all of these complications (Lenke and Levy 1980; Rohr et al. 1987; Koch et al. 2003). The International Maternal PKU Collaborative Study (MPKUCS), a prospective, longitudinal study, showed that this was especially true if dietary therapy with control of maternal phenylalanine began before pregnancy or within the first 6 weeks of gestation (Koch et al. 2003.) In the MPKUCS, 228 children who were born to mothers with treated PKU or untreated mild hyperphenylalaninemia were compared to 70 control subjects at 7 years of age. Offspring cognitive outcome negatively correlated with the number of gestational weeks that elapsed until maternal metabolic control was achieved ($r = -0.61$). Behavioral outcome was similarly affected. Postnatal measurement of stimulation in the home was also related to offspring IQ (Waisbren and Azen 2003). However, the MPKUCS followed offspring only to age 7 years, thus was not able to evaluate cognitive performance into the more challenging school years.

The ability of the mother with PKU to provide a secure and intellectually stimulating environment for her offspring needs further examination. Poor metabolic control in adults with PKU is associated with deficits in executive functioning, including planning, organization, and behavioral inhibition as well as fatigue, health problems, depression, and anxiety (Koch et al. 2010; Brumm et al. 2010), all of which may limit parenting ability and result in a suboptimal environment for the offspring.

In this study, we examined offspring, ages 1 month to 26 years, from treated maternal PKU pregnancies and analyzed associations between maternal factors, including current adherence to recommendations for treatment, and offspring outcomes, such as cognitive abilities and emotional/behavioral characteristics.

Methods

Participants

Families with children in which the mother has PKU requiring dietary treatment (excluding non-PKU mild hyperphenylalaninemia) were invited to participate in this study. Classification of degree of PKU in the mothers was established as set forth by the European multicenter study (Guldberg et al. 1998) and was based on at least two of the following indictors: confirmatory or untreated blood phenylalanine level; dietary tolerance for phenylalanine; and phenylalanine hydroxylase (PAH) genotype. The study was approved by the institutional review board (IRB) of Boston Children's Hospital. The parents and children in each family were seen in the Clinical and Translational Study Unit (CTSU) of Boston Children's Hospital. The mother in each family as well as all offspring above the age of 6 years gave informed consent/assent. Nine fathers consented to having a photo taken for comparison of offspring physiognomy.

Evaluations

Clinical

A detailed health history was obtained from the mothers and a health and developmental history was obtained on the offspring. The mothers and offspring received a general physical examination as well as a neurologic assessment. Dysmorphology in offspring was evaluated through measurements of inner and outer canthal distances, interpupillary distance, lengths of the palpebral fissures, and the lengths of both ears (Hall et al. 2007). Frontal and side photographs of the face were obtained on the offspring and, for comparison, on the mothers and fathers. Parental photographs allowed for identification of dysmorphic features associated with maternal PKU and not simply reflective of familial characteristics.

Nutrition

Mothers were interviewed about their current diets (including a questionnaire about the use of medical foods, avoidance of protein, and other nutrition therapies) and asked to provide a 24 h diet recall. Laboratory studies included plasma amino acids, hemoglobin and hematocrit, prealbumin, 25-hydroxy vitamin D, red blood cell folate, iron, ferritin, zinc, and vitamin B_{12}. The mother's

adherence to diet during pregnancy was assessed by the review of pregnancy blood phenylalanine results, and assignment was made into one of three categories: (1) Excellent: on diet prior to conception, blood phenylalanine <360 μmol/L throughout pregnancy; (2) Good: on diet prior to conception, blood phenylalanine <360 μmol/L by 10 weeks gestation; (3) Fair: blood phenylalanine <600 μmol/L by second trimester; and (4) Poor: on diet after conception and blood phenylalanine not in control until the second trimester or after.

Neuropsychological

Mothers were administered the Wechsler Abbreviated Scale of Intelligence (WASI) (Wechsler 1999). They also completed self-report measures of adaptive behavior (Adaptive Behavior Assessment System—Second Edition (ABAS-II)) (Harrison and Oakland 2003), executive functioning (Behavior Rating Inventory of Executive Functioning (BRIEF)) (Gioia et al. 2000), anxiety (Beck Anxiety Index (BAI)) (Beck and Steer 1993), and depression (Beck Depression Index—Second Edition (BDI-II)) (Beck et al. 1996).

The offspring were administered age-appropriate measures of intellectual development: Bayley Scales of Infant and Toddler Development, Third Edition (Bayley 2003), for children less than 36 months of age; Wechsler Preschool and Primary Scales of Intelligence, Third Edition (WPPSI-III) (Wechsler 2002), for ages 4–6 years; and the WASI for offspring ages 7 years and older. In addition, the offspring completed the Beery-Buktenica Developmental Test of Visual Motor Integration, Sixth Edition (VMI) (Beery et al. 2010), which measures visual-spatial abilities and fine motor coordination. The mothers rated their children using age-appropriate forms of the ABAS-II, BRIEF, and a measure of emotional well-being, Behavior Assessment System for Children, Second Edition (BASC-II) (Reynolds and Kamphaus 2003).

Statistical Analyses

We report descriptive statistics for the offspring and mother characteristics and outcomes, including mean and standard deviation or median and range as appropriate. Two-group comparisons of continuous variables use the Wilcoxon rank-sum test of median values, and comparisons of categorical variables use Fisher's exact test. For distributional reasons, we report Spearman's correlation coefficients for rank associations of continuous variables. Because of multiple child observations per mother, we use a Monte Carlo bootstrap approach with 10,000 iterations to calculate 95% confidence intervals (CIs) and p-values for correlations of child and maternal outcomes. Similarly we used linear models fit with generalized estimating equations (GEE) to compare mean values of child outcomes between groups.

Table 1 Sample demographics

	Mean ± SD, range or *n* (%)
Mothers (n = 24)	
Age (years)	39 ± 7, 24–49
Education (years)	14 ± 3, 9–18
Hollingshead–Redlich social position	39 ± 19, 15–73
Marital status	
Married	19 (79)
Single	3 (13)
Divorced	2 (8)
Offspring (n = 48)	
Mean ± SD age (years)	8.5 ± 6.2, 0–26
Ages 0–3 years (infancy)	12 (26)
Ages 4–5 years (preschool)	6 (13)
Ages 6–18 years (school age)	26 (55)
Ages 19+ years (adult)	3 (6)
Sex	21 males (44)
	27 females (56)

Results

Sample Demographics

The 24 mothers were well into their childbearing years (Table 1) and 10/24 (42%) completed a bachelor's degree in college. The majority were married and had middle-class socioeconomic status. The Hollingshead Index of Social Position (Hollingshead 1957) ranged from 15 to 73, where low scores indicate higher levels of education and employment. In our sample, 9/23 (39%) families were in the lowest two Hollingshead social positions (scores >43).

There were 77 pregnancies among the mothers with 56/77 (73%) of pregnancies resulting in live births (including 2 sets of twins), 16/77 (21%) spontaneous abortions, and 5/77 (6%) terminations. Of the 58 live births, one offspring suffered asphyxia at birth, with associated cerebral palsy, and was excluded from the study since her condition was judged by her physicians as not related to her mother's PKU. One offspring with PKU was included since he had been in excellent metabolic control since birth. An additional 10/58 (17%) were unavailable due to living in another state, working or no longer in contact with the mother. A total of 47 offspring (81%) were available for study. The majority were of school age or older, with 29/47 (62%) ages 6–18 years. Also included in the sample were

Table 2 Offspring cognitive and emotional outcomes

Outcome	Mean ± SD	Range	*n* (%) outside normative range[a]
Bayley scales of infant and Toddler development, third edition (ages < 3 years)			
Bayley cognitive	113 ± 12	95–130	0/9 (0)
Bayley language composite	112 ± 12	94–132	0/9 (0)
Bayley motor composite	101 ± 11	82–124	1/9 (11)
Intelligence test (WPPSI-III or WASI) (ages > 3 years)[b]			
Full Scale IQ	94 ± 19	53–138	9/36 (25)
Verbal IQ	94 ± 20	55–150	12/36 (33)
Performance IQ	97 ± 17	56–127	7/36 (19)
Vocabulary subtest (WASI)	45 ± 13	20–69	8/24 (33)
Block design subtest (WASI)	48 ± 11	23–65	4/24 (17)
Similarities subtest (WASI)	44 ± 12	20–66	8/24 (33)
Matrix reasoning subtest (WASI)	48 ± 14	20–65	5/24 (21)
Visual-spatial skills			
Visual motor integration test (VMI)	89 ± 16	45–118	10/33 (30)
Adaptive behavior assessment system, second edition (ABAS-II)			
General adaptive composite (GAC)	97 ± 19	43–130	9/45 (20)
Conceptual	99 ± 20	49–133	11/46 (24)
Social	102 ± 17	61–133	8/46 (17)
Practical	92 ± 18	42–120	11/46 (24)
Behavior assessment system for children, second edition (BASC-2)			
BASC somatization	46 ± 11	33–69	6/38 (16)
BASC attention problems	50 ± 10	33–76	6/38 (16)

[a] Thresholds indicating performance within normative range on neuropsychological measures:
– >85 for BSID, IQ, VMI, and ABAS-II scales
– >40 for WASI subtest scores
– <60 for BASC-2
[b] 24 offspring received a WASI and 12 preschool children received the WPPSI-III

12 infants (<3 years), 6 preschool-aged children (ages 4–5 years), and 3 young adults (ages 19–26 years). Among the 47 offspring, 18 (38%) were from pregnancies in which adherence to medical recommendations was rated as excellent, 15 (32%) in which adherence was good, 10 (21%) in which adherence was fair, and 4 (9%) in which adherence was poor.

Offspring Outcomes

Dysmorphology

The only facial dysmorphology noted in the offspring was hypertelorism; the mean percentile distances were 73 ± 25% for the inner canthal distance, 89 ± 12% for outer canthal distance, and 83 ± 22% for the interpupillary distance. Other features of facial dysmorphology that have been reported in treated MPKU such as epicanthal folds, elongated and smooth philtrum, and high-arched palate were not observed. Comparisons between facial photographs of maternal PKU offspring and their parents revealed no other dysmorphic findings. None of the offspring had congenital heart disease. The mean head circumference of the offspring was in the 48th percentile but with the wide range of <3–99%. Nine (19%) of the 47 offspring had head circumference <3 percentile, indicating microcephaly.

Cognitive Functioning and Emotional Well-Being

Child cognitive and emotional outcomes are presented in Table 2. The mean Cognitive, Language, and Motor Composite scores on the Bayley Scales of Infant and Toddler Development, Third Edition, were well within the average range (85–115). Infants received a mean DQ of 113 ± 12, while preschool children attained a mean IQ of 96 ± 19 and older children attained the same mean Full Scale IQ of 96 ± 19. The mean IQ for the three adults in the study was 81 ± 15, considerably lower than the IQ of the school-aged children. The correlation between age and

Full Scale IQ in offspring over age 3 years was -0.32 ($p = 0.06$). Overall, 6/26 (23%) offspring attained a Full Scale IQ in the borderline range (IQ 70–85) and 3/26 (12%) performed in the range of intellectual disabilities (IQ < 70). Difficulties in fine motor coordination and visual-spatial skills were noted on the VMI, for which the child is asked to copy a series of increasingly complex geometric figures. The mean score on the VMI was 89 ± 16, with 10/33 children (30%) at least one standard deviation below the normal mean of 100.

The mean score on the ABAS-II General Adaptive Composite (GAC) was comparable to Full Scale IQ. The mother's responses indicated that children had relative weaknesses in the practical domain and strengths in the social realm.

Scores on the Behavior Assessment System for Children, Second Edition, were generally within the average range. These mothers perceived their children as well adjusted and well behaved. The only two scales with moderately elevated scores were Somatization (physical complaints) and Attention Problems, both with 6/38 (16%) in the at-risk range.

However, the mother's answers to direct questions related to school functioning and medical intervention suggest that maternal PKU offspring experience troublesome symptoms not readily detected by intelligence testing or the parent questionnaires we administered. Among the children at least 5 years of age, 8/32 (25%) had learning disabilities, 10 had attention deficit hyperactivity disorder (ADHD) (31%) with 7 on ADHD medication (22%), and 11 had been diagnosed with anxiety and/or depression (34%). These percentages are above rates reported for the general population in which 1.9% of children are known to have learning disabilities (Brault 2012), 5.1% are labeled as having ADHD (2014a), 6.5% are on medication for ADHD in Massachusetts (ADHD 2014b), and 15–20% of children suffer from anxiety or depression (Beesdo et al. 2009). In addition, two children in our sample had been diagnosed with bipolar disorder, and one teenaged boy suffered from substance abuse.

Maternal Outcomes

Maternal Health and Nutrition

Among the 24 mothers, 16 (67%) had severe PKU, 6 (25%) had moderate PKU, and 2 (8%) had mild PKU. History and physical examination of the mothers did not reveal any major abnormalities. One mother had a papular erythematous rash on both arms, another mother had recurrent basal cell carcinomas, and a third mother had mild eczema. Among 22 mothers whose height and weight were measured, 5 (23%) had body mass index (BMI) in the normal range, 10 (45%) were overweight, 6 (27%) were obese, and 1 (5%) was underweight. All 24 women answered the questionnaire about diet practices, and 12 (50%) reported following a diet for PKU although 14 (58%) reported taking a PKU formula/medical food. Among 12 women responding to a question about formula consumption, 9 (75%) reported taking at least 75% of the amount of prescribed medical food. Overall, 16/23 mothers (70%) reported restricting protein intake.

Of the 10 women who reported being off-diet, 3 (30%) stated that they restricted protein, and one of them also reported taking medical food. Among the entire sample, 3/24 women (13%) were treated with tetrahydrobiopterin (sapropterin dihydrochloride or Kuvan®), a cofactor for phenylalanine hydroxylase. No women were treated with large neutral amino acid (LNAA) therapy.

Laboratory indices of maternal nutritional intake are presented in Table 3. Results are stratified by self-report of phenylalanine-restricted diet. Laboratory values for phenylalanine, tyrosine, vitamin B12, and RBC folate differed significantly between the on- and off-diet groups. While the on-diet group had significantly lower blood phenylalanine compared to the off-diet group, overall only 3/23 mothers (15%) had blood phenylalanine levels less than 600 μmol/L at time of the study and none had blood phenylalanine <360 μmol/L.

Vitamin D was abnormally low in 1/3 (33%) of on-diet mothers and 5/7 (71%) of those off-diet. Prealbumin values were predominantly in the normal range, suggesting adequate protein intake. Hemoglobin and hematocrit values were normal and did not differ significantly between the groups. Vitamin B12 and RBC folate were significantly higher in the on-diet group.

Maternal Cognitive and Emotional Outcomes

As noted in Table 4, mean scores were within the average range (85–115) on the Wechsler Abbreviated Scale of Intelligence (WASI). The mean maternal Full Scale IQ was 94 ± 16, with 6/24 mothers (25%) having a Full Scale IQ < 85 (one standard deviation below the population normal mean.) Maternal Performance IQ was on average 9.4 points higher than Verbal IQ, reflecting reduced vocabulary and verbal reasoning abilities ($p = 0.01$, 95% CI 2.7–16.1 points higher).

The ABAS-II measures self-reported functioning in a variety of domains, including cognitive, social and practical. The mothers rated themselves slightly above the population norm of 100 on all dimensions, with a mean overall score of 110 ± 10. Among the 23 mothers completing this questionnaire, only 1 (4%) received a score <85, which on this test similarly represents more than one standard deviation below the population mean.

Table 3 Median and range of dietary intake and laboratory findings in mothers with PKU on and off a phenylalanine-restricted diet (figure in parenthesis indicates number of women with values out of recommended range)

	On-diet ($n = 12$)	Off-diet ($n = 10$)	Reference values	p-value[a]
Phenylalanine (μmol/L)	816; 389–1,610 (10/12)	1,319; 395–1,934 (9/10)	120–600[b]	0.04
Tyrosine (μmol/L)	47; 30–112 (1/12)	32; 25–36 (5/9)	32–122	0.03
Prealbumin (g/dL)	27; 23–43 (1/12)	25; 20–35 (0/10)	20–40	0.64
Vitamin D (ng/mL)	40.2; 25.3–43.4 (1/3)	28.7; 21.2–39.2 (5/7)	30–80	0.34
Vitamin B12 (pg/mL)	844.5; 197–1,336 (6/12)	328; 223–859 (0/9)	211–946	0.02
Ferritin (mg/dL)	53.5; 18–108 (0/12)	67; 15–198 (2/9)	13–150	0.11
Hemoglobin (g/dL)	13.5; 12.4–14.3 (0/12)	13.2; 12.3–14.7 (0/9)	11.5–16	0.52
Hematocrit (%)	39.5; 35.8–43.1 (0/12)	38.5; 35.6–41.4 (0/9)	34–44	0.27
Plasma Zinc (ng/mL)	119; 58–193 (4/12)	128.5; 94–158 (1/8)	70–150	0.42
RBC folate (ng/mL)	922; 579–1,468 (3/12)	828; 417–862 (1/9)	468–1,258	0.03

[a] p-values from the Wilcoxon rank-sum test
[b] Recommended blood phenylalanine range for adults with PKU

Table 4 Maternal neuropsychological outcomes

	Mean ± SD	Range	Outside normal bounds[a] (%)
All mothers			
Full Scale IQ[b]	94 ± 16	61–117	6/24 (25)
Verbal IQ	90 ± 16	59–113	7/18 (39)
Performance IQ	100 ± 16	69–126	3/18 (17)
ABAS GAC	110 ± 11	83–128	1/23 (4)
ABAS Conceptual	106 ± 18	39–120	2/23 (9)
ABAS Social	104 ± 21	26–120	4/23 (17)
ABAS Practical	108 ± 14	63–120	1/23 (4)
BRIEF GEC	46 ± 10	35–76	1/23 (4)
Beck depression inventory	8.3 ± 9.8	0–39	6/24 (25)
Beck anxiety inventory	6.7 ± 7.0	0–30	11/24 (46)
Mothers on formula			
Full Scale IQ	100 ± 11	79–117	1/14 (7)
Verbal IQ	96 ± 13	80–109	3/9 (33)
Performance IQ	105 ± 14	83–126	1/9 (11)
Beck depression inventory	5.8 ± 7.2	0–26	2/14 (14)
Beck anxiety inventory	3.9 ± 4.3	0–12	5/14 (36)
Mothers not on formula			
Full Scale IQ	86 ± 19	61–115	5/10 (50)
Verbal IQ	85 ± 18	59–113	4/9 (44)
Performance IQ	95 ± 18	69–123	2/9 (22)
Beck depression inventory	11.7 ± 12.1	0–39	4/10 (40)
Beck anxiety inventory	10.6 ± 8.3	0–30	6/10 (60)

ABAS GAC Adaptive Behavior Assessment System, General Adaptive Composite; *BRIEF GEC* Behavior Rating Inventory of Executive Function, Global Executive Composite
Thresholds for normal neuropsychological test measures used:
– >=85 for IQ measures
– <=64 for BRIEF GEC
– <=13 for Beck Depression Inventory
– <=7 for Beck Anxiety Inventory
[a] Six mothers received the 2-subtest form of the WASI, which yields only a Full Scale IQ

The mothers did not report themselves as having difficulties in executive functioning, as measured by the BRIEF. Of the 23 mothers completing this self-report questionnaire, 1 (4%) received a score >65 on the Global Executive Composite (GEC).

Emotionally, a different picture emerged, with 6/24 mothers (25%) receiving scores on the self-report Beck Depression Inventory, Second Edition (BDI-II) > 13, indicating mild to moderate depression, and 11/24 mothers (46%) reporting mild to moderate anxiety on the Beck Anxiety Inventory (BAI) with scores >7. These percentages are higher than reported in the general population, where annual rates are 9.5% for depression and 18.1% for anxiety (Kessler et al. 2005).

Mean scores on all measures of cognitive functioning were directionally higher for mothers on-diet (defined as taking formula). Moreover, 2/14 on-diet mothers (14%) self-reported depression on the BDI-II compared to 4/10 off-diet mothers (40%) (Fisher $p = 0.19$), and 5/14 on-diet mothers (27%) self-reported anxiety on the BAI compared to 6/10 off-diet mothers (60%) (Fisher $p = 0.41$).

Correlations Between Offspring Outcome and Maternal Characteristics

As expected, offspring IQ correlated highly with maternal metabolic control during pregnancy ($r = 0.51$, $p = 0.002$, 95% CI (0.19, 0.74)), maternal IQ ($r = 0.62$, $p = 0.0001$, 95% CI (0.33, 0.81)), and the Hollingshead Index of Social Position (Hollingshead 1957) ($r = -0.48$, $p = 0.005$, 95% CI (−0.68, −0.12)). Offspring IQ was not associated with current maternal blood phenylalanine level ($r = -0.10$, $p = 0.55$, 95% CI (−0.44, 0.25)), diet status (on- or off-diet) (mean difference 0.6, $p = 0.96$), or maternal marital status (mean difference 4.6, $p = 0.38$).

Children whose mothers had depression or anxiety were at risk for behavioral difficulties, psychiatric problems, and learning disabilities: 7/11 (64%) of children with anxiety or depression, 6/10 (60%) of children with ADHD, and 6/8 (75%) children with learning disabilities had mothers with anxiety or depression.

Discussion

The purpose of this study was to obtain a longer-term assessment of offspring from treated MPKU pregnancies and to identify maternal and environmental characteristics associated with offspring outcome. Published results of offspring outcome from treated maternal PKU have been limited to developmental studies of the neonatal and early infancy periods or to cognitive assessments in the first few years of childhood. The oldest offspring from treated maternal PKU pregnancies in our previous New England study was 4 years old (Rohr et al. 1987), and the evaluations of offspring in the MPKUCS ended at age 7 years (Koch et al. 2003; Waisbren and Azen 2003). In this study, 62% of the offspring were older than age 5 years with the oldest age 26 years. This study extended the length of follow-up into adolescence and early adulthood and also examined emotional and behavioral characteristics of these offspring. Particular attention was given to those factors of PKU in the mothers that might affect their ability to provide a secure and stimulating environment for their offspring.

None of the maternal PKU offspring in our study had evidence of heart disease or a history of having had congenital heart disease. The mean head circumference of the 48th percentile was within the expected range for the general population although in several instances, MPKU might have had a lingering adverse effect on head growth since 19% had a head circumference below the 3rd percentile, lower than the 31% for microcephaly among offspring in the MPKUCS. Facial dysmorphology included evidence of hypertelorism, as previously reported (Rouse et al. 1997, 2000), but other facial features reported as due to MPKU were absent. In summary, we found growth and somatic development in the offspring similar to the general population.

Despite the variability in current adherence to the diet for PKU, the majority of women who participated in this study perceived themselves as functioning well in their daily lives. Nonetheless, 25% performed in the borderline intellectual range, much higher than the 12.3% reported in epidemiological studies (e.g., Hassiotis et al. 2008) and higher than the 15.9% expected based on the normal population curve for IQ. Their mean IQ was 94, lower than that of adults with PKU recently studied in Europe (Weglage et al. 2013). In the study of 57 adults with PKU, the mean IQ was 100.6 compared to an IQ of 110.4 in a matched control group. However, the group of mothers in our study taking their medical food (formula) was 100, the same as that in the European study. Nearly 40% of mothers in our study were in the two lowest categories of socioeconomic position. Closer examination of their scores on tests of verbal expression, verbal reasoning, depression, and anxiety revealed vulnerabilities that may have an important impact on their parenting skills and hence on their children's development and well-being.

Within the general population, individuals with IQ in the borderline range are significantly more likely to be at social disadvantage, experience neurotic disorders (such as anxiety and depression), and suffer from substance misuse. They take psychotropic medications and seek emergency services at a higher rate but are not more likely to seek psychotherapy

(Hassiotis et al. 2008). Other studies report that adults with borderline IQ are at risk for poor occupational attainment and depression (Seltzer et al. 2009).

Children of parents with borderline IQ, anxiety, or depression also seem to be negatively affected, not so much during the preschool period but after age 7 years, with increased rates of conduct disorders and emotional and attention problems (Whitely et al. 2011). Likewise in our study of maternal PKU offspring, children under age 3 years exhibited fewer cognitive or behavioral deficits than children older than 3 years. By school age, the children were much more likely to have ADHD, learning disabilities, and anxiety or depression than children in the general population. This suggests that the deficits noted later in childhood may be related not only to prenatal effects but also to environmental circumstances, including the home environment, maternal depression, anxiety, and educational opportunities. We found that the majority of school-aged maternal PKU offspring with behavioral disturbances, emotional difficulties, or low IQ had mothers who were depressed or anxious. The percentage of children with psychiatric symptoms or low IQ may be even higher than we reported, given that one mother appeared to be an "outlier." Her 5 offspring had a range of problems including anxiety and depression, ADHD, low IQ, and learning disabilities, but she rated herself as having no anxiety or depression. Based on clinical observation and prior medical reports, however, she experiences both anxiety and depression. If she had rated herself as such, all offspring with ADHD, all with low IQ, all with learning disabilities, and 82% of those with anxiety and depression would have mothers who were experiencing anxiety or depression.

While there was overlap between current maternal blood phenylalanine results for the on- and off-diet groups, median blood phenylalanine concentrations were lower in the on-diet group. Notably, only one woman on diet had blood phenylalanine concentration <360 μmol/L, which is considered to be safe for an MPKU pregnancy (Vockley et al. 2014). The typical "on-diet" approach for this population consisted of taking about 75% of the prescribed phenylalanine-free or low-phenylalanine medical food (formula) for PKU and avoiding high-protein foods such as meat, eggs, nuts, and dairy products. Few women strictly reduced their phenylalanine intake, as evidenced by elevated plasma phenylalanine concentrations.

The women off-diet were at significantly higher risk of having low blood tyrosine, which may be counterintuitive since the off-diet group consumes more natural protein. However, medical food is a source of tyrosine and may account for the significantly higher blood tyrosine in the on-diet group. Other discrepancies between amino acid values in the two groups are notable, especially higher blood concentrations of the large neutral amino acids—valine, leucine, and tryptophan—in the on-diet group. Valine and leucine are large neutral amino acids (LNAAs) which compete for transport of phenylalanine into the brain. High amounts of LNAAs in the blood block phenylalanine uptake as well as promote neurotransmitter synthesis (Pietz et al. 1999).

Half of the women had 25-OH vitamin D results below the lower limit of 30 ng/mL, but only one had severe vitamin D deficiency (<20 ng/mL) (Christesen et al. 2012). A recent Cochrane review indicates that vitamin D supplementation and higher levels of 25-OH vitamin D in pregnant women have been associated with increased birth weight. Moreover, observational studies show a positive effect of vitamin D status on other health outcomes in children. Indices of iron nurtiture (hemoglobin, hematocrit, ferritin) were normal in both groups, as were zinc and folate. Plasma vitamin B12 was significantly lower in the off-diet group compared to the on-diet group, again indicating that the medical food is a significant source of vitamin B12 in the diets of mothers with PKU. Vitamin B12 deficiency in adults with PKU is a well-established phenomenon arising from diets that do not contain either medical food or animal products (Hvas et al. 2006).

The prevalence of overweight women with PKU (45%) and obesity (27%) was somewhat above national norms that indicate 64% of the population is either overweight or obese Fryar and Ogden 2014; Fryar et al. 2014. Female children with PKU have been reported to have a higher incidence of being overweight or obese in a single study (Burrage et al. 2012). In a previous study of pregnant women, a bimodal distribution of prepregnancy weight was observed with nearly equal numbers of overweight and underweight women (Rohr et al. 2004).

The suboptimal nutritional status of these women with PKU may have implications for their emotional well-being. For example, recent studies demonstrate associations between low vitamin D or low vitamin B12 and depression (Anglin et al. 2013; Kalita et al. 2013), and low tyrosine has been implicated in depressive symptoms in PKU (Sharman et al. 2012). Moreover, anxiety and agoraphobia have long been recognized as symptoms of poor metabolic control in PKU patients (Waisbren and Levy 1991; Brumm et al. 2004).

Very few women were being treated with tetrahydrobiopterin supplementation and none with large neutral amino acids. The women who were enrolled in the study were older, and many were off-diet and not seen frequently in clinic. Thus, they may be part of the "lost generation" of adults with PKU who do not have the opportunity to learn about updated therapies for PKU (Burton and Leviton 2010). However, this MPKU follow-up study offered an opportunity to update women about treatment options, and yet none chose to change their current treatment. Responses

to the nutrition questionnaire may provide insight into this behavior. About half of the women who were off-diet reported that they had tried to return to diet at some point in the postpartum period. When asked about obstacles to returning to diet, the most common responses were not the expected ones (difficulty with formula, protein restriction, or insurance) but rather that they did not perceive a need for any treatment. Only one mother reported lack of access to treatment as an obstacle.

One limitation of this study was the use of self-report and parental report instruments. Rates of offspring difficulties may be underestimated in our study, since the mothers tended not to report themselves or their children as functioning below the average range while cognitive test scores tended to suggest otherwise. Subgroup comparisons of on-diet versus off-diet women or children with versus without cognitive deficits or mood disorders involved small sample sizes.

Conclusion

The primary conclusion of this study is that maternal diet influences offspring outcome—before, during, and after pregnancy. Mothers who are well treated from the very beginning of their lives have a higher IQ and therefore often a higher SES, both of which were found to correlate with offspring outcome. They are also less likely to be anxious or depressed which was a determining factor in offspring with ADHD, anxiety, depression, and low IQ. While maternal metabolic control during pregnancy explains much about offspring outcomes, other parental characteristics may also contribute to the increased rates of low IQ and attention problems in these children, and these appear to be directly related to diet and medical food intake. Moreover, it takes time for the maternal PKU offspring's problems to emerge. Most social-emotional problems were not evident until the children were school-aged. For this reason, it is important that MPKU offspring have psychological evaluations throughout childhood and adolescence and that mothers continue to receive therapy for PKU throughout the lifespan. Evidence for positive maternal PKU pregnancy outcomes is now well established. This study illustrates that risks associated with maternal PKU do not end with the birth of the infant, but continue throughout the child's life. To ensure optimal offspring outcomes, healthcare providers need to assess maternal nutrition, blood phenylalanine concentrations, cognitive abilities, mood, and socioeconomic position. Interventions can then be initiated that reduce psychosocial stressors and enhance adherence to medical recommendations and positive parenting, which in turn can lead to better cognitive functioning, behavior, and emotional well-being in the children.

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Compliance with Ethics Guidelines

Conflict of Interest

We wish to draw the attention of the editor to the following facts which may be considered as potential conflicts of interest and to significant financial contributions to this work.

Dr. Susan Waisbren receives grant support from BioMarin Pharmaceuticals and has, in the past, consulted to the company with regard to psychological assessment of individuals with PKU. She also receives funds from the National Institutes of Health for the study of genomic sequencing in newborn screening.

Dr. Harvey Levy receives grant support from BioMarin Pharmaceuticals for a Phase 3 clinical trial of PEG-PAL enzyme therapy for PKU and for a PKUDOS study of outcome of Kuvan therapy in PKU. He also receives funds from the National Institute of Health for a Phase 2 crossover trial of glycomacropeptide in dietary therapy for PKU and for the study of genomic sequencing in newborn screening.

Frances Rohr, Vera Anastasoaie, Matthew Brown, Dr. David Harris, Al Ozonoff, Stephanie Petrides, and Ann Wessel have no conflicts of interest to disclose.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki

Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from Susan.Waisbren@childrens.harvard.edu.

Author Contributions

Dr. Waisbren conceived and designed this study. She oversaw data collection, conducted data analyses, interpreted data and drafted the manuscript.

Frances Rohr, MS, RD, LDN assisted in data collection, analysis and interpretation. She contributed to the manuscript drafts.

Vera Anastasoaie, BA assisted in data collection, analysis and interpretation. She critically reviewed the manuscript.

Matthew Brown, BA assisted in data analysis and interpretation and critically reviewed the manuscript.

David Harris, MD assisted in data collection, analysis and interpretation and contributed to the writing and review of the manuscript.

Al Ozonoff: Assisted in data analysis and interpretation. He critically reviewed and revised the manuscript.

Stephanie Petrides, BA assisted in data collection, analysis and interpretation. She critically reviewed the manuscript

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RESEARCH REPORT

Assessing Psychological Functioning in Metabolic Disorders: Validation of the Adaptive Behavior Assessment System, Second Edition (ABAS-II), and the Behavior Rating Inventory of Executive Function (BRIEF) for Identification of Individuals at Risk

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Abstract Long-term follow-up of neuropsychological functioning in metabolic disorders remains difficult due to limited opportunities for comprehensive neuropsychological evaluations. This study examined the validity of using the Adaptive Behavior Assessment System, Second Edition (ABAS-II), and the Behavior Rating Inventory of Executive Function (BRIEF) for assessing developmental status in metabolic disorders and for identifying individuals at risk for cognitive deficits. Results from individuals with urea cycle disorders, phenylketonuria, galactosemia, and fatty acid oxidation disorders were obtained on the ABAS-II and BRIEF and were compared to results obtained from neuropsychological testing performed on the same day. Correlations between scores on the ABAS-II and developmental or IQ tests for individuals with urea cycle disorders ranged from 0.48 to 0.72 and concordance rates for scores greater than a standard deviation below the normative mean ranged from 69 to 89%. Correlations ranged from 0.20 to 0.68 with concordance ranging from 73 to 90% in the other metabolic disorders. For the BRIEF, correlations with other tests of executive functioning were significant for urea cycle disorders, with concordance ranging from 52 to 80%. For the other metabolic disorders, correlations ranged from −0.09 to −0.55. Concordance rates for at-risk status on the BRIEF and executive functioning tests ranged from 55% in adults to 80% in children with other metabolic disorders. These results indicate that the ABAS-II and BRIEF together can confidently be used as an adjunct or supplementary method for clinical follow-up and for research on functional status involving infants, children, and adults with metabolic disorders.

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Introduction

In spite of the risks to development, children and adults with metabolic disorders may not receive neuropsychological evaluations as part of routine care in the metabolic clinic. This situation occurs because of limited access to a psychologist who specializes in metabolic disorders and because of challenges to obtaining insurance approval for assessments. Consequently, individuals with these disorders often go years without a neurodevelopmental or neuropsychological evaluation and thus receive needed interventions only after symptoms become severe and are far more difficult to treat. Moreover, research into the neuropsychological effects of metabolic disorders is hindered by the lack of uniform follow-up assessments.

Recognition of the need for a method for assessing psychological functioning and for gathering a standard data set in metabolic disorders led a team of 10 psychologists and a psychiatrist to select instruments to serve as a Uniform Assessment Method. The goal was to develop validated, short, and inexpensive standardized protocols

that would be relevant throughout the life span, could be completed by parents or the affected adults, could be available in Spanish as well as English, and could be administered, scored, and interpreted by non-psychologists. In addition, instruments were chosen for their power to identify children or adults in need of further evaluation, treatment modifications, or closer monitoring (Waisbren and White 2010). Among the instruments selected were the Adaptive Behavior Assessment System, Second Edition (ABAS-II) (Harrison and Oakland 2003), and the Behavior Rating Inventory of Executive Function (BRIEF) (Gioia et al. 2000). Other instruments included were the Behavior Assessment System for Children – Second Edition (BASC-2) (Reynolds and Kamphaus 2004) and, for adults, the Beck Depression Inventory, Second Edition (Beck et al. 1996), and the Beck Anxiety Inventory (Beck and Steer 1993). While the questionnaires selected were already well validated in the general population, their psychometric properties had not been described for individuals with metabolic disorders. The purpose of this Uniform Assessment Method is not to find a substitute for intelligence testing or comprehensive neuropsychological evaluations but rather to establish a quick, valid, and feasible means to assess functioning for routine clinical follow-up, registries, and research studies.

In this report, results from the ABAS-II and BRIEF obtained on individuals with urea cycle disorders (UCDs), phenylketonuria (PKU), galactosemia, and fatty acid oxidation disorders (FAODs) were compared to results obtained from neuropsychological testing performed on the same day. PKU, galactosemia, and FAODs were selected for comparison to UCDs because they represent metabolic disorders with neuropsychological impact for which comparable test results were available from other research studies or medical records. We did not include the BASC-2 or the Beck Depression or Anxiety Inventories because they were not routinely available in the research databases or medical records.

Metabolic Disorders

Urea Cycle Disorders

UCDs interfere with the hepatic ammonia detoxification pathway, leading to hyperammonemia and other biochemical abnormalities. Absence or deficiency of the first four enzymes in the urea cycle (carbamoyl phosphate synthetase I (CPSI), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL)) leads to hyperammonemia within the first few days of life. Deficiency or absence of arginase 1 (ARG1) leads to other neurological and developmental problems, although hyperammonemia is not as common (Summar et al. 2008). Ornithine transcarbamylase (OTC) deficiency, the most common of the urea cycle disorders (OMIM 311250), is an X-linked inherited disorder, in which males are more severely affected. Many females remain asymptomatic throughout their lives, although subtle neuropsychological deficits arise that can interfere with functioning in day-to-day life (Gyato et al. 2004; Gropman et al. 2010). The clinical symptoms related to these disorders are variable, ranging from neonatal death due to complications of hyperammonemia to normal cognitive and developmental outcomes throughout life (Krivitzky et al. 2009). Newborn screening has recently become available for several of the urea cycle disorders (Beck et al. 2011), and while early diagnosis and treatment may reduce the frequency and severity of hyperammonemic episodes (Summar 2001), they do not eliminate risks of reduced cognitive abilities, maladaptive behaviors, and poor executive functioning (Krivitzky et al. 2009; Ah Mew et al. 2013).

Phenylketonuria (PKU)

PKU (OMIM 261600) is an autosomal recessive disorder that affects the body's ability to metabolize phenylalanine into tyrosine, the precursor of the neurotransmitter, dopamine. Untreated PKU is associated with severe intellectual disabilities, seizures, eczema, motor difficulties, and significant behavioral problems. Fortunately, universal, mandatory newborn screening in the United States and in most developed countries around the world permits the identification and early treatment of this inborn error of metabolism. Treatment consists of a low-protein diet and a supplemental formula that provides all the necessary parts of protein without the "offending" amino acid (Scriver and Kaufman 2001). With early treatment, most children with PKU develop well, with intellectual abilities within the average range (Waisbren et al. 2007). However, many individuals experience executive functioning deficits (Christ et al. 2010), attention deficit disorder (Antshel and Waisbren 2003), and psychiatric problems, including anxiety and depression (Brumm et al. 2010).

Galactosemia

Galactosemia (OMIM 230400) is a rare, inherited metabolic disorder in which the metabolism of galactose is impaired, due to insufficiency or absence of the enzyme, galactose-1-phosphate (Fridovich-Keil and Walter 2008). If left untreated, classic galactosemia can cause severe neonatal sepsis, cataracts, and death. Those who survive the newborn period often experience intellectual disability, speech and language delay, and motor deficits. With early identification through newborn screening and treatment with a galactose-restricted diet, the more severe consequences of this disorder are prevented. However, almost every

child with galactosemia has speech/language delay, and many have minor to moderate motor deficits (Bosch 2006). Adults also exhibit motor deficits, depression, and anxiety (Waisbren et al. 2012).

Fatty Acid Oxidation Disorders

Mitochondrial fatty acid oxidation is a complex process involving transport of activated acyl-coenzyme A (CoA) moieties into the mitochondria and sequential removal of two carbon acetyl-CoA units. This process in the mitochondria provides energy for many tissues including heart and skeletal muscle and is critically important during times of fasting or physiologic stress (Vockley and Whiteman 2002). Disorders of fatty acid oxidation, the most common of which is medium-chain acyl-CoA dehydrogenase deficiency (OMIM 607008), interrupt this cycle and lead to a deficit in the conversion of fat into energy. Most patients with fatty acid oxidation defects are now identified through newborn screening, and as a result, mortality and morbidity rates have vastly improved (Wilcken 2010). However, developmental delays are not uncommon, particularly in the area of speech and language (Iafolla et al. 1994; Waisbren et al. 2013).

This summary of metabolic disorders exposes the continued neuropsychological and behavioral risks confronting patients with these conditions. Without adequate developmental and neuropsychological screening and follow-up, their needs often remain unrecognized.

Instruments

The Adaptive Behavior Assessment System, Second Edition (ABAS-II) (Harrison and Oakland 2003), spans the entire life span, from early infancy to adulthood. The ABAS-II is a checklist of a broad range of skill areas related to development, behavior, and cognitive abilities. Parents or other informants can complete the age-appropriate form (0–5 years, 6–21 years). For capable adults there is a self-report form, as well as a parent/informant version. The ABAS-II includes subscales for communication, community use, functional academics, home living, health and safety, leisure, self-care, self-direction, social, and work. A scaled score of 10 ± 3 represents the mean. Four composite scores are derived from the sum of the scaled scores: general adaptive composite (GAC), conceptual, social, and practical. These composite scores have a mean of 100 and a standard deviation of 15. The ABAS-II standardization sample included 1,670 respondents. The ABAS-II GAC has a correlation of 0.54 with the Wechsler Preschool and Primary Scale of Intelligence (WPPSI-III) and 0.41 with the Wechsler Intelligence Scale for Children, Fourth Edition (WISC-IV). Computerized scoring and interpretation programs are now available. This questionnaire takes 10–15 min to complete.

The Behavior Rating Inventory of Executive Function (BRIEF) (Gioia et al. 2000) provides theoretically and empirically derived clinical scales that measure aspects of executive function. Executive functioning can be thought of as the ability to keep information in mind for problem solving. It involves such cognitive processes as memory, attention, planning, organization, and the ability to shift attention from one thought to another. The clinical scales form broad indices of behavior and cognition and an overall score, the global executive composite (GEC). For this study, parent response forms for school-aged children were analyzed, although there is also a preschool version. A self-report form and an informant response form are available for adults, permitting a uniform measure across all ages. All forms were standardized on normative samples representing a broad variety of race/ethnicity, age, and geographical population density. A T score of 50 ± 10 represents the mean of the T-score distribution, and a score of 65 represents 1.5 standard deviations above the mean, which is the recommended cut point for an "abnormally elevated" score and is considered "clinically significant." The questionnaire is completed within 10–15 min.

Methods

This study incorporated information from research data sets and medical records. Parents of children with urea cycle disorders or adults with urea cycle disorders participating in a longitudinal study completed the ABAS-II and BRIEF as part of comprehensive neuropsychological evaluations (Seminara et al. 2010; Krivitzky et al. 2009; Ah Mew et al. 2013). Additional reports on results from this study are forthcoming. Data on children with PKU, galactosemia, and fatty acid oxidation disorders were obtained from a review of medical records from Boston Children's Hospital, where the questionnaires from the Uniform Assessment Method have been used for the past 5 years at the time of routine psychological evaluation. (See Waisbren et al. 2013, for a review of results on children with FAODs.) Data on adult women with PKU were obtained from a study of maternal PKU (Waisbren et al. in press) that included administration of the ABAS-II, BRIEF, and intelligence testing. Data on adults with galactosemia were obtained from a study that also included the ABAS-II, BRIEF, and neuropsychological assessment (Waisbren et al. 2012). Approval for these various studies was obtained by the Boston Children's Hospital Committee on Clinical Investigations (IRB) or other metabolic centers where the

research was being conducted. Approval was also received to conduct a medical record review of Boston Children's Hospital patients.

Mean and standard deviations were calculated to describe the scores on the ABAS-II, BRIEF, developmental tests, intelligence tests, and scales that measured aspects of executive functioning. T tests and analysis of variance were used to evaluate differences in scores between males and females and across the different disease groups. We categorized results according to the following age groups: infants (<3 years), preschool children (3–5 years), school-aged children (6–17 years), and adults (18+ years).

Results from subjects with PKU, galactosemia, and fatty acid oxidation disorders were combined into an "other metabolic disorders" group, due to relatively few cases with these disorders. This "other metabolic disorders" group was compared to results from subjects with UCDs. For 41 cases in the urea cycle group, a parent or informant completed the ABAS-II for adult subjects. Analyses were performed with and without these cases, and occasionally a difference in results was noted. Occasionally, subjects were rated more than once on the ABAS-II or BRIEF at the time of a neuropsychological evaluation, and their data were included in the analyses. Results from the BRIEF were derived only from school-aged children and adults.

Pearson correlation coefficients were computed to assess strength of relationships between the ABAS-II and measures of cognitive functioning. For infants, the GAC from the ABAS-II was compared to the cognitive composite score from the Bayley Scales of Infant and Toddler Development, Third Edition (Bayley 2005). For older children and adults, the ABAS-II GAC was compared to the full-scale IQ obtained from the Wechsler Preschool and Primary Scales of Intelligence, Third Edition (WPPSI-III) (Wechsler 2002), the Wechsler Intelligence Scale for Children, Fourth Edition (WISC-IV) (Wechsler 2003), the Wechsler Adult Intelligence Scale, Fourth Edition (WAIS-IV) (Wechsler 2008), or the Wechsler Abbreviated Scale of Intelligence (WASI) (Wechsler 1999), depending on the age of the subject and instrument used for clinical or research purposes. Pearson correlations were also calculated to assess the strength of associations between the BRIEF Global Executive Composite (GEC) and two scales from instruments that measure aspects of executive functioning, specifically working memory as measured by the California Verbal Learning Test (CVLT), Trial 5 (Delis et al. 2000; Tremont et al. 2000), and planning as measured by the block design subtest from the Wechsler intelligence tests (Weiss et al. 2006).

Concordance (agreement) between the ABAS-II and developmental or intelligence tests provided a measure of validity of the ABAS-II to identify children at risk and in need of further evaluation. A score of 85 (1 standard deviation below the published mean) represented the cut point for determining a risk for problems in adaptive functioning on the ABAS-II General Adaptive Composite (GAC) and for risk of developmental delay or intellectual deficits on the Bayley Cognitive Composite and full-scale IQ on the WPPSI-III, WISC-IV, WAIS-IV, or WASI.

Concordance between the BRIEF GEC and the two scales measuring aspects of executive functioning was also determined. For the BRIEF GEC, the cut point for executive functioning deficits is ≥65, as specified by the BRIEF manual. On the CVLT trial 5, a score one standard deviation below the mean (≤ −1.0) represented risk for executive functioning deficits. For the block design subtest from the Wechsler scales of intelligence, the cut point for risk of deficits was set at 7 or below (or ≤40 on the WASI). In addition to measuring agreement, percents for receiver operating characteristic (ROC) area under the curve (AUC) were calculated.

Results

As noted in Table 1, 516 individuals were rated on the ABAS-II and 265 individuals were rated on the BRIEF. More females than males were included in the study. The mean age of the sample was 13.8 years but ranged from infancy to middle age. Differences in scores on the ABAS-II or BRIEF between males and females were noted only when parent ratings of adults with UCDs on the ABAS-II were included with the self-ratings of adults with UCDs ($p < 0.04$).

The mean score on the ABAS-II GAC was lower for the combined UCD cases (86 ± 23) than for the combined other metabolic disorders (mean = 99 ± 16, $p < 0.0001$). Similarly, scores on the BRIEF were higher (indicating greater difficulties in executive functioning) for the UCD cases (mean = 57 ± 12) compared to the cases with other metabolic disorders (mean = 53 ± 13, $p < 0.01$). Significant differences were not noted on the ABAS-II or BRIEF among individuals with PKU, galactosemia, or FAODs.

As noted in Table 2, mean scores on the ABAS-II GAC were within the low-average to high-average range for all study groups as were mean scores for the Bayley Cognitive Composite and full-scale IQ. The correlations between the ABAS-II GAC and the Bayley Cognitive Composite in infants or full-scale IQ in children and adults ranged from 0.20 to 0.70, with the correlations consistently high among school-aged children 6–17 years of age. For children and adults with urea cycle disorders, mean scores on the two measures were within 1–4 points. For children with other metabolic disorders, mean scores derived from parent ratings were 9 to 18 points lower than scores on tests administered directly (Bayley Scales or Wechsler intelli-

Table 1 Total number and mean age of individuals with each disorder rated on the ABAS-II and BRIEF and number of males and females

Disorder	ABAS-II[a]	ABAS-II[b]	BRIEF	Mean age ± standard deviation	Female	Male
	N				*N* (% of total)	*N* (% of total)
Urea cycle disorders	282	323	174	11.2 ± 11.0	208 (67%)	115 (56%)
Phenylketonuria	94	94	56	19.8 ± 17.6	66 (21%)	28 (14%)
Galactosemia	68	68	24	18.6 ± 15.4	30 (9%)	38 (19%)
Fatty acid oxidation disorders	31	31	11	5.2 ± 3.7	8 (3%)	23 (11%)
Totals	475	516	265	13.8 ± 13.9	312 (100%)	204 (100%)

[a] ABAS-II using only self-report for adults
[b] ABAS-II using self-report for adults when available, supplemented by parent reports in the absence of a self-report

Table 2 Mean (standard deviation) scores on the ABAS-II general adaptive composite (GAC), Bayley cognitive composite, correlations with IQ tests, and concordance with scores below the cut point for the ABAS-II GAC

Neurocognitive instrument	*N*	ABAS-II GAC	Bayley cognitive composite	IQ	Pearson correlation (*r*)	Confidence interval for *r*	Concordance[a]
UCD							
Children <3 years	105	87 ± 19	89 ± 19		0.48	(0.32,0.62)	73/105 (69%)
Children 3–5 years	50	86 ± 20		91 ± 20	0.67	(0.48,0.80)	37/50 (74%)
Children 6–17 years	132	83 ± 22		89 ± 21	0.64	(0.53,0.73)	93/13 (70%)
Adults 18+[b]	47	105 ± 13		103 ± 15	0.54	(0.30,0.72)	42/47 (89%)
Adults 18+[c]	86	98 ± 18		95 ± 21	0.72	(0.59,0.80)	74/86 (86%)
Other metabolic disorders							
Children <3 years.	34	100 ± 12	118 ± 17		0.21	(−0.16, 0.53)	27/30 (90%)
Children 3–5	40	97 ± 16		108 ± 12	0.20	(−0.12, 0.48)	34/40 (85%)
Children 6–17	44	90 ± 19		101 ± 17	0.68	(0.47, 0.81)	37/44 (84%)
Adults 18+	75	103 ± 15		93 ± 18	0.42	(0.22, 0.59)	55/75 (73%)

[a] Concordance includes all test results from each participant. Cut points for ABAS-II, Bayley cognitive composite, and full-scale IQ ≤ 85
[b] UCD using only self-report for adults
[c] UCD using self-report for adults when available, supplemented by parent reports in the absence of a self-report

gence tests), despite generally high correlations. For adults with the other metabolic disorders, the opposite was true, with mean self-rated ABAS-II GAC 10 points higher than full-scale IQ.

For the UCD cases, the communication subscale from the ABAS-II correlated with the Bayley Language Composite score ($r = 0.60$, $p < 0.000001$). The motor subscale from the ABAS-II correlated with the Bayley Motor Composite score ($r = 0.57$, $p < 0.00001$). These data were not available for subjects with the other metabolic disorders.

Table 3 presents comparisons between the BRIEF global executive composite (GEC) and scores on two tests that measure aspects of executive functioning (working memory and planning). Among adults with UCDs, confidence intervals for the correlations indicate that the GEC was associated with working memory as measured by the CVLT. For children and adults with UCDs, the BRIEF GEC also correlated with scores on block design, measuring planning and organization. Although correlations were high between the GEC and aspects of executive functioning for subjects with other metabolic disorders, these associations did not reach significance. In general, individuals with UCDs exhibited deficits on both the CVLT and block design. For individuals with the other metabolic disorders, scores on block design remained relatively intact, while particular vulnerabilities were noted in working memory, as measured by the CVLT.

For the ABAS-II GAC, concordance refers to agreement with the Bayley Cognitive Composite or full-scale IQ in terms of a score above or below the cut point indicative of risk for developmental delay or cognitive deficit. As noted in Table 2, overall concordance ranged from 69 to 90%.

Table 3 Pearson correlations between BRIEF questionnaires global executive composite (GEC) and tests of executive functioning by study group and age and concordance with scores above the cut point for the BRIEF and below the cut point on the block design or CVLT

Study/age group	*N*	BRIEF GEC mean (SD)	Test *N*, mean (SD)	Pearson correlation	Confidence interval for *r*	Concordance[a]
UCD						
6–17 years	112	58 ± 12	CVLT	−0.16	(−0.34, 0.025)	74/112 (66%)
			−0.37 ± 1.39			75/143 (52%)
	143	60 ± 12	Block Design* 7.2 ± 3.9	−0.35	(−0.49, −0.20)	
18+ years	43	53 ± 10	CVLT	−0.42	(−0.64, −0.14)	30/43 (70%)
			−0.48 ± 1.28			38/60 (63%)
	60	54 ± 11	Block Design 6.7 ± 4.3	−0.38	(−0.58, −0.14)	
Other metabolic disorders						
6–17 years	10	61 ± 14	CVLT	−0.55	(−0.88, 0.12)	8/10 (80%)
			−0.35 ± 1.1			23/35 (64%)
	35	58 ± 13	Block design 10 ± 3	−0.09	(−0.41, 0.25)	
18+ years	9	54 ± 9	CVLT −1.67 ± 1.62	−0.55	(−0.89, 0.18)	5/9 (55%)
	29	49 ± 10	Block design 9 ± 4	−0.26	(−0.58, 0.11)	19/29 (66%)

[a] Cut points: BRIEF $\geq$ 65; CVLT $\leq$ −1.0; block design $\leq$7 (scores on block design from the WASI were converted to scaled scores)

As noted in Table 3, concordance rates between the BRIEF GEC and either the CVLT or block design ranged from 52 to 80%, with agreement generally higher for the CVLT, measuring working memory, than for block design, measuring planning as well as visual spatial abilities.

For the ABAS-II GAC, ROC AUC summarizing overall sensitivity and specificity is 69% for infants with UCDs and 24% for infants with other metabolic disorders. The ROC AUC is 80% or higher for school-aged children and above 70% for adults with UCDs and other metabolic disorders. For the BRIEF GEC, the ROC AUC is 60% for school-aged children with UCDs and 84% for school-aged children with other metabolic disorders. For adults, it is above 60% for both individuals with UCDs and individuals with other metabolic disorders.

Discussion

In this study, 515 individuals with metabolic disorders contributed data from the ABAS-II and 265 contributed data from the BRIEF. Data from these questionnaires were compared to scores obtained through neurodevelopmental or neuropsychological testing. This study focused on UCDs, galactosemia, PKU, and FAODs because data on these populations were available. The assessment method proposed here could easily be applied to the many other metabolic disorders that also present at various ages and are associated with a broad range of psychological outcomes. Mean scores on the ABAS-II GAC and the Bayley Cognitive Composite or IQ tests from individuals with urea cycle disorders were within 4 points. For the other metabolic disorders, the ABAS-II GAC mean scores tended to be lower than the Bayley Cognitive Composite or full-scale IQ for children, but higher than full-scale IQ for adults.

The correlations between the ABAS-II GAC and full-scale IQ ranged from 0.62 to 0.68 in school-aged children, considerably higher than the correlation between the GAC and the WISC-IV full-scale IQ ($r = 0.41$) reported in the ABAS-II manual. When discrepancies occurred in this study, parent ratings usually indicated problems, whereas the cognitive composite or full-scale IQ was within the average range. A review of medical records and scores on other neuropsychological tests suggested that nearly all these children had attention deficits, hyperactivity, or other behavioral problems. These children received higher scores on an IQ test than on the ABAS-II, which identifies potential behavioral as well as cognitive risk factors.

Strong correlations were noted among scores from UCD individuals between the BRIEF GEC and block design for school-aged children and adults. The GEC was also highly

correlated with the CVLT for adults with UCDs. Scores on the BRIEF from individuals with the other metabolic disorders were not significantly correlated with scores on the CVLT or block design. With test scores from only 9 to 35 individuals, this aspect of the study may have been underpowered.

The analyses for this study were limited to the summary scores on the ABAS-II and BRIEF. The subscales for both these instruments may prove to be valid as additional outcome measures. However, assessment of these subscales was beyond the scope of this study, which relied on existing data sets.

It is important to remember that adaptive functioning, as assessed by the ABAS-II, is not equivalent to intellectual functioning, since it describes a much broader range of behaviors, including social relationships, self-help skills, and ability to get along in the community. Similarly, executive functioning as measured by the BRIEF is not equivalent to isolated skills of memory and planning but includes attention, inhibition, organization, and other abilities. Given that the potential bias is in the direction of overidentification of at-risk children, assessments based on the ABAS-II and BRIEF will be unlikely to miss a child who has a developmental delay or deficit in executive functioning.

Ideally, the ABAS-II and BRIEF will be incorporated in every metabolic disorder study that includes measures of functioning so that results can be compared across studies and meta-analyses can be easily conducted. Uniform assessments with the ABAS-II and BRIEF can overcome the problems of small sample sizes and insufficient resources when examining functioning in newborn screening or when conducting long-term follow-up studies.

Use of the ABAS-II and BRIEF for assessing every child identified with a metabolic disorder will increase the likelihood of insurance coverage for further evaluations when needed and permit early identification of those requiring early interventions or treatment modifications. Moreover, inclusion of anonymous results from these measures in longitudinal registries will provide critical data for evaluating the efficacy of newborn screening, which has expanded greatly in the past decade and is likely to increase further as new screening technologies become available.

These instruments are available as paper forms or online and can be administered on a tablet or laptop computer. They can be administered by non-psychologists. They extend across all age groups and exist in Spanish and other languages as well as English. Reports can be generated electronically, and scores can be entered into databases for research purposes or tailored for feedback to patients, families, and health-care providers.

Future studies are needed to evaluate the sensitivity of the ABAS-II and BRIEF as pre- and posttests in medication or other treatment trials. Additional studies are needed to validate the other tests recommended in the Uniform Assessment Method described by Waisbren and White (2010), including the BASC, Beck Anxiety Inventory, and Beck Depression Inventory to assess psychiatric and emotional well-being.

In conclusion, the results of this study indicate that the ABAS-II and BRIEF together can confidently be used as an adjunct or supplementary method for clinical follow-up and for research on functional status in infants, children, and adults with metabolic disorders. This method can serve as an "early warning" system to detect neuropsychological deficits for which a full evaluation would be important. Ideally, the ABAS-II and BRIEF can be administered as part of regular follow-up for metabolic disorders to monitor treatment effectiveness and disease progression. While comprehensive neuropsychological evaluations provide a more differentiated picture of the types and severity of deficits associated with these disorders, the expense, time commitment, and limited access to such testing has prohibited and probably always will prohibit its use as a method for routine follow-up in clinical settings or for measures of functional status in registries or large research studies. The ABAS-II and BRIEF can fill this void.

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BioMarin Pharmaceutical Company and the Galactosemia Foundation provided support for the studies from which data on individuals with PKU and galactosemia were extracted.

One-Sentence Synopsis

The Adaptive Behavior Assessment System, Second Edition (ABAS-II), and the Behavior Rating Inventory of Executive Function (BRIEF) are two questionnaires that provide a valid and quick method for assessing psychological functioning in urea cycle and other metabolic disorders.

Compliance with Ethics Guidelines

Conflict of Interest

Dr. Waisbren receives grant support from BioMarin Pharmaceuticals and has, in the past, consulted to the company with regard to psychological assessment of individuals with PKU.

Dr. He and Dr. McCarter declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5).

Informed consent was obtained from all patients included in the studies. The Institutional Review Board at Boston Children's Hospital approved the medical record review from patients who were not enrolled in a study but who had received the questionnaires and neuropsychological testing as part of clinical follow-up in the past.

Contributions

Dr. Waisbren conceived and designed this study. She oversaw data collection, conducted data analyses, interpreted data, and drafted the manuscript.

Dr. He assisted in data analysis and interpretation. He critically reviewed and revised the manuscript.

Dr. McCarter assisted in study design and was the lead contributor to data analysis and interpretation. He assisted in initial drafting of the manuscript and critically reviewed and revised subsequent drafts.

Guarantor: Susan Waisbren, Ph.D.

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The authors confirm independence from the sponsors; the content of the article has not been influenced by the sponsors.

Institutional Review Board (IRB) approval was obtained for each of the studies from which data were extracted. All subjects with urea cycle disorders signed informed consent forms as part of their participation in the Longitudinal Study of Urea Cycle Disorders. Permission to conduct a chart review and use existing databases from studies in phenylketonuria, galactosemia, and fatty acid oxidation disorders for which informed consent had previously been obtained was granted by the IRB at Boston Children's Hospital.

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RESEARCH REPORT

Glutaric Acidemia Type 1-Clinico-Molecular Profile and Novel Mutations in *GCDH* Gene in Indian Patients

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Abstract Glutaric acidemia I (GA I, #231670) is one of the treatable, autosomal recessively inherited metabolic disorders. Macrocephaly, acute encephalitis-like crises, dystonia and characteristic frontotemporal atrophy are the hallmarks of this disease. In this communication, we present the clinical, biochemical and molecular profile of seventeen GA I patients from 15 unrelated families from India and report seven novel mutations in *GCDH* gene (c.281G>A (p.Arg94Gln), c.401A>G (p.Asp134Gly), c.662T>C (p.Leu221Pro), c.881G>C (p.Arg294Pro), c.1173dupG (p.Asn392Glufs*5), c.1238A>G (p.Tyr413Cys) and c.1241A>C (p.Glu414Ala)). Out of these, c.662T>C (p.Leu221Pro) in exon 8 and c.281G>A (p.Arg94Gln) allele in exon 4 were low excretor alleles, whereas c.1241A>C (p.Glu414Ala), c.1173dupG and c.1207C>T (p.His403Tyr) in exon 11 were high excretor alleles. We conclude that c.1204C>T (p.Arg402Trp) is probably the most common mutant allele. Exons 11 and 8 are the hot spot regions of *GCDH* gene in Indian patients with GA I. An early diagnosis and timely intervention can improve the underlying prognosis. Molecular confirmation is helpful in providing genetic counselling and prenatal diagnosis in subsequent pregnancy.

The original version of this chapter contained errors which have been corrected. These are detailed in the erratum to be found under 10.1007/8904_2015_448.

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Introduction

Glutaric acidurias are a group of autosomal recessively inherited metabolic disorders that are characterized by abnormal excretion of glutaric acid due to a defect in amino acid or fatty acid metabolism pathways. Glutaric acidemia type 1 or glutaric aciduria type 1 (GA I) occurs due to the deficiency of glutaryl-CoA dehydrogenase (GCDH) enzyme (EC 1.3.8.6, old number EC 1.3.99.7), a member of the acyl-CoA dehydrogenase family and a key enzyme in the catabolic pathways of the amino acids tryptophan, lysine and hydroxylysine. Deficiency of GCDH causes increased organic acid excretion of glutaric acid, 3-hydroxyglutaric acid and glutaconic acid in urine and

elevated glutarylcarnitine (C5DC) in plasma. The estimated worldwide frequency of GA I is 1 in 100,000 newborns (Lindner et al. 2004), but increased frequency has been reported in the inbred Old-Order Amish community of Pennsylvania (Morton et al. 1991) and the Ojibway Indians in Manitoba (Haworth et al. 1991). About ninety percent of the affected children present classically between 2 and 37 months of age with extrapyramidal symptoms, predominantly dystonia superimposed on axial hypotonia after an acute encephalopathic crisis precipitated by intercurrent febrile illness, infection, fasting or immunization (Hoffmann et al. 1991; Kolker et al. 2006; Strauss et al. 2003). About 75% of the patients have macrocephaly with soft neurological signs such as head lag, irritability and feeding difficulties. Extrapyramidal symptoms are due to bilateral striatal injury during the acute episode. Early diagnosis and prompt initiation of treatment can prevent the long-term complications and mortality which have led to its inclusion in conservative newborn screening programs (Heringer et al. 2010; Kolker et al. 2007). GCDH enzyme is encoded by *GCDH* gene, which is located on the chromosome 19p13.2 spanning about ~7 kb and contains 12 exons (Transcript ID ENST00000222214) and encodes 438 amino acid-long precursor protein. It is a genetically heterogeneous condition and is caused by different types of mutations such as missense, nonsense and intronic variations in *GCDH* gene. Till date, 163 mutations have been reported from different ethnic groups in HGMD (Goodman et al. 1998; Busquets et al. 2000a; Zschocke et al. 2000). Although there is some correlation between genotype and the urinary excretion of glutaric acid, the correlation between genotype and phenotype has been elusive (Kolker et al. 2006). There is no published data from India on the mutation spectrum of GA I. This retrospective study is aimed to present clinical and molecular profile of Indian patients and report seven novel mutations. In silico analysis was performed to predict the role of these novel mutations on protein function. The effects of novel missense mutations on the structure of gene product have been analyzed by computational modelling. Prenatal diagnosis was offered in one family.

Materials and Methods

Patient Enrolment

Over a period of 5 years (June 2008 till June 2013), 17 patients of varying age from 15 unrelated families were recruited from the Genetic Clinic and Pediatric Neurology Clinic. The study was approved by the institutional ethics committee, and written informed consent was taken from all parents. Clinical details were filled in a predesigned proforma for neurometabolic disorders. Diagnosis was made based upon the clinical presentation, neuroimaging, urinary organic acids and tandem mass spectrometry results. Wherever possible, clinical outcome was assessed through follow-up either clinically and by review of records or through parents' interview where the child was not alive. During follow-up assessment, patients were evaluated mainly clinically and not biochemically for amino acids due to lack of availability of amino acids and affordability. Age-dependent developmental assessment tools were used for the developmental delay. It included calculation of developmental age and quotient using the Developmental Assessment Scale for Indian Infants (DASII) for 0–30 months, Developmental Profile 3 (DP 3) for 0–12 years and Malin's Intelligence Scale for Indian Children (MISIC) for 6–15 years. No formal scoring for the severity of movement disorder or morbidity was used.

GCDH Gene Analysis

Peripheral blood was collected in EDTA vacutainer, and genomic DNA was isolated by salt method (Miller et al. 1988). Primers were designed by primer 3 software (bioinfo.ut.ee/primer 3–0.4.0) (Supplementary Table 1), and coding sequences were amplified in eight different fragments by polymerase chain reaction (PCR). Amplified PCR products were purified by enzyme treatment (ExoI & SAP) and sequenced by ABI 3130 Genetic Analyzer (Applied Biosystem, USA). Sequence analysis was performed by using ChromasPro software (http://technelysium.com.au) and NCBI blast. Nomenclature of novel mutations was done according to the guidelines of Human Genome Variation Society (http://www.hgvs.org/mutnomen). Novel mutations were checked in the 1000 Genome Projects database (http://www.1000genomes.org/), dbSNP database (http://www.ncbi.nlm.nih.gov/snp/) and HGMD (Human Gene Mutation Database, http://www.hgmd.org/).

In Silico Analysis of Novel Mutations

Multiple sequence alignments were performed to check the conservation of amino acid across some other species. PolyPhen2 (http://genetics.bwh.harvard.edu/pph2) and SIFT (http://sift.jcvi.org) programs were used to assess the qualitative effect of missense changes on the protein function. Both these programs predict the effect of amino acid replacement due to mutation on the function of protein. PolyPhen2 predicts the effect based on physicochemical properties of residue and comparative analysis, while SIFT predicts on the basis of degree of conservation of residues through PSI-BLAST sequence alignment.

Three-dimensional structure of the proteins is required to understand the role of mutations on their structure and

function at atomic level. Crystal structure of wild-type human GCDH complexed with coenzyme FAD (PDB: 1SIQ) (Fu et al. 2004) is available in PDB. Model structure of protein with novel missense mutation was built with the help of crystal structure of wild-type protein and "Build Mutant" protocol incorporated in MODELLER 8.2 program (Sali and Blundell 1993) in the modelling environment of Discovery Studio (DS 2.0) (Discovery Studio 2006, Accelrys Inc., San Diego, CA, USA). The "Build Mutant" protocol changes the selected residue into the desired residue and optimizes the conformation of mutated residue along with neighbouring residues. Side chains of the residues in the models were further refined to correct their conformation and orientation using the modelling program "Chi Rotor" (Spassov et al. 2007). The stereochemical quality of the mutant models was verified by PROCHECK (Laskowski et al. 1993). The generated models were energy minimized to remove any steric clash between atoms, followed by short MD simulations for the final refinement using CHARMm force field simulation engine (Brooks et al. 1983; Momany and Rone 1992).

Results

A total of fifteen unrelated families and seventeen patients were recruited during the study period. The male/female ratio was 1.4:1. The median age at onset of symptoms and final diagnosis was 6 months (2−12 months) and 18 months (3–192 months), respectively. Out of 15 families, 3 had positive family history. Consanguinity was seen in 8/15 (53%) families with different religious backgrounds (5 Muslim, 1 South Indian, 2 Hindu). Twelve patients (70%) had precipitating illness like fever associated with seizure (4/12), fever associated with/without gastroenteritis or pneumonia (4/12), seizure alone (3/12) or after immunization (1/12). The main presenting features were macrocephaly, developmental regression mainly motor due to development of dystonia, seizures or a combination of these. Table 1 shows the distribution of clinical, biochemical and radiological abnormalities across various families. Patients were divided into four developmental categories: patients with (1) normal development, (2) developmental regression, (3) developmental delay only and (4) developmental delay with regression category. "Regression" in GA I essentially refers to loss of intentional motor control due to development of dystonia. Six patients (35.2%) had normal initial development followed by regression and dystonia, two had normal development without regression or dystonia but had associated seizures and one had normal development with dystonia. The rest of the eight patients (47%) had developmental delay with or without regression with associated extrapyramidal symptoms in all except one. Based upon the developmental assessment by DASII, DP3 and MISIC at different ages, all patients except F2, F10a, F12 and F15 had delay in the physical and adaptive domain primarily because of the development of dystonia. Patients F7, F9 and F15 had mild global developmental delay, whereas patients F1, F3, F4, F8 and F13 had severe to profound delay.

Macrocephaly, dystonia, choreoathetoid movements and seizures were present in 70.5% (12/17), 64.7% (11/17), 0.05% (1/17) and 52.9% (9/17), respectively. Patients with generalized tonic–clonic seizures had initial one or two episodes followed by complete disappearance. Three patients had myoclonic seizures. Both striatal (hyperintensities in caudate and putamen) and extrastriatal (subdural hygroma, wide sylvian fissure, frontotemporal atrophy, hyperintensities in globus pallidus and thalamus) abnormalities on neuroimaging were seen in all the patients. Majority of the patients had feeding difficulties especially in swallowing. Soon after diagnosis, all the patients were kept on indigenous lysine- and tryptophan-restricted diet (predominantly a vegetarian Indian (native/local) diet in natural form) and carnitine supplementation (50–100 mg/kg/day) due to nonavailability and affordability of GA I-specific diet. Lysine restriction was achieved by natural and locally available diet. The amount of lysine varied depending upon the different age groups with an average daily intake of 90–120 mg/kg body weight/day, the highest being in infants. The total protein intake was 1–2 g protein/kg actual body weight/day. All patients were also supplemented with riboflavin (100–300 mg/day), calcium and other micronutrients soon after the diagnosis, and none of them reported to have any major deficiencies. These patients were on naturally achieved lysine-restricted vegetarian diet, and lysine-free, tryptophan-reduced amino acid supplements were not given separately due to nonavailability of these amino acid supplements. Baclofen (10–20 mg/day) was used for dystonia. Two patients (F3 and F5) died at the age of 1.6 and 2.6 years, respectively. Both these children had severe feeding difficulties and were malnourished. They died at home probably due to aspiration pneumonia.

GCDH Gene Mutations

A total of 15 different mutations were identified in *GCDH* gene in different exons in 17 patients of 15 unrelated families. Seven novel (c.281G>A (p.Arg94Gln), c.401A>G (p.Asp134Gly), c.662T>C (p.Leu221Pro), c.881G>C (p.Arg294Pro), c.1173dupG (p.Asn392Glufs*5), c.1238A>G (p.Tyr413Cys) and c.1241A>C (p.Glu414Ala)) and eight reported (c.650C>T (p.Pro217Leu), c.769C>T (p.Arg257Trp), c.764C>T (p.Ser255Leu), c.770G>A (p.Arg257Gln), c.937C>T (p.Arg313Trp), 1173delG (p.Asn392Metfs*9), c.1204C>T (p.Arg402Trp) and c.1207C>T (p.His403Tyr))

Table 1 Distribution of clinical, biochemical and radiological abnormalities across various families

Case number	Gender	Age at onset (months)	Age at diagnosis (months)	Precipitating illness	Developmental category ($n = 17$)	Macrocephaly	Extrapyramidal symptoms	Seizures	Abnormal neuro radiological changes[a]	Elevated glutaryl carnitine (C5 DC)	Elevated urine elevated glutaric acid and its metabolites	Outcome
F2	M	2	3	Fever, acute gastroenteritis, seizures	Normal development (3)	+	–	+	+	+	+	Alive
F10a	F	6	132	Seizure, vomiting		+	–	+	+	+	+	Alive
F6	M	4.6	5	Seizures		–	+	+	+	+	+	Alive
F5	F	6	18	Fever, seizures	Regression[b] (6)	+	+	+	+	+	+	Died
F14	F	6	7	Fever, seizures		+	+	+	+	+	+	Alive
F10b	M	5	24	Fever, gastroenteritis		+	+	–	+	+	+	Alive
F11a	F	7	24	Fever		–	+	–	+	+	–	Alive
F11b	M	6	12	Seizures once only		–	+	–	+	–	–	Alive
F12	M	7.6	10	No		+	–	+[c]	+	+	+	Alive
F9	F	12	192	No	Developmental delay (4)	+	+[d]	+[c]	+	+		Alive
F1	M	10	54	No		+	+	–	+	+	+	Alive
F4	M	6	10	Pneumonia		+	–	–	+	+	+	Alive
F15	M	6	30	No		+	–	–	+	+	+	Alive
F7	M	7	60	Fever, seizures	Developmental delay and regression[b] (4)	+	+	+	+	+	+	Alive
F8	F	6	36	Injection		–	+	–	+	+	+	Alive
F3	F	6	6	Fever, seizures		+	+	–	+	+	+	Died
F13	M	8	10	No		–	+	+[c]	+	+	+	Alive

[a] *Extrastriatal* neuroradiological abnormalities
Temporal hypoplasia/frontotemporal atrophy, wide temporal and sylvian CSF spaces (bat-wing appearance) in F2, F4, F6, F7, F8, F9, F10b, F12, F15
Subdural hygroma F2, F4, F10a, F12
Hyperintensities in globus pallidus (GP)] and thalamus (T) – F4 (GP), F8 (T), F13 (GP)
Striatal neuroradiological abnormalities – hyperintensities in caudate (C), putamen (P), F1 (P), F2 (C,P), F3 (C,P), F4 (P), F5 (P,C), F6 (P), F8 (P), F10b (C,P), F11a (P), F15 (C,P)
[b] Loss of intentional motor control
[c] Myoclonic seizures
[d] Chorieform movements

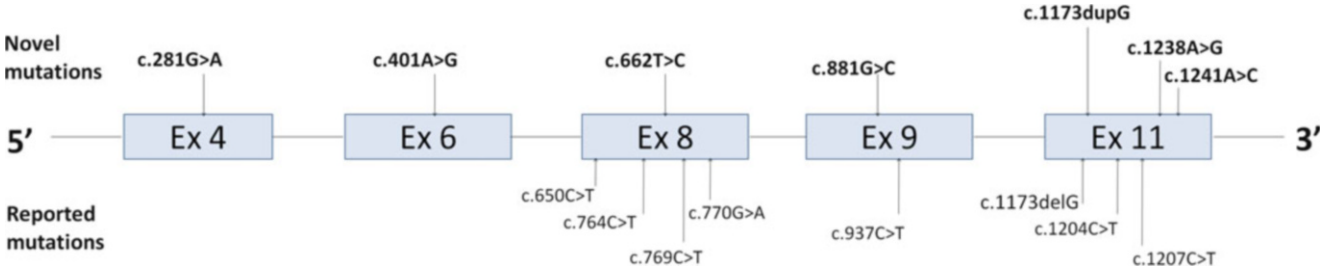

Fig. 1 Mutation distribution across *GCDH* gene

mutations (Fig. 1, Table 2) were found only in five different exons of *GCDH* gene. Mutation c.1207C>T (p.His403Tyr) was initially thought to be novel in nature, but recently it has been reported (Wang et al. 2013). Amongst the seven novel mutations, one is frameshift and six are missense mutations. Prenatal diagnosis was performed for one family (F1). Upon counselling, pregnancy was terminated as the foetus was affected.

In Silico Analysis

All the novel mutations were found in the highly conserved region of the protein. Both PolyPhen2 and SIFT programs predict the effect of observed novel mutation in this study to have deleterious effect on the function of protein. Stereochemical qualities of mutant models are good, and substituted residues lie in the most favoured region of Ramachandran's plot. In silico study through the three-dimensional structure of the model of the mutant proteins helps to understand the effect of mutation on the structure of protein.

Genotype and Biochemical Phenotype

Patients were divided into high excretors and low excretors based upon the criteria by Baric et al. (1999) and Kolker et al. (2006). High excretors were defined as glutaric acid excretion of >100 mmol GA/mol creatinine and low excretors as <100 mmol GA/mol creatinine. Levels of glutaric acids were available only in six patients with five novel alleles. In our study, out of seven novel alleles, two alleles, c.662T>C (p.Leu221Pro) and c.281G>A (p.Arg94Gln), had almost no glutaric aciduria (F11a and b) and hence were low excretors; however, c.1241A>C (p.Glu414Ala) (F2), c.1173dupG (F12) and c.1207C>T (p.His403Tyr) in Ex-11 (F13, F14) were found to be high excretor alleles.

Discussion

There have been few reports of GA I in Indian patients, the majority being on neuroimaging (Kamate et al. 2012; Sen and Pillay 2011). This is the first report on clinical, biochemical and molecular profile of Indian patients.

Clinical Spectrum

Glutaric aciduria type I is probably the second common organic acidemia in India, the commonest being methylmalonic acidemia (unpublished data, personal communication). These patients have a variable course of illness, the majority of patients presenting with loss of intentional motor control due to development of dystonia precipitated by an acute febrile illness, macrocephaly and extrapyramidal symptoms. In our cohort, the median age at the onset of symptoms was 6 months. The age at diagnosis was quite variable and there was a gap of nearly 12 months between the onset and diagnosis probably because of the variable clinical spectrum of the disorder or due to the delayed diagnosis. Twelve out of 17 (70%) had some precipitating factor at around 6 months. Out of these three had normal development, while others had developmental delay and/or regression. In accordance with other studies (Wang et al. 2013; Mushimoto et al. 2011), a combination of macrocephaly, developmental regression, dystonia and seizures emerged as the predominant presenting feature. Three out of 17 had normal development (F2, F10a, F6) despite having the precipitating event and radiological abnormalities. Case F2 was diagnosed at 3 months and kept on modified diet and carnitine supplementation. He has good compliance and is developmentally normal. All patients with or without crises had MRI abnormalities (Harting et al. 2009). Eighty two percent of the patients (14/17) were already neurologically compromised (categories 2–4) at the time of presentation. Category 2 patients had mainly motor regression due to incapacitating dystonia. A correlation between MRI findings and dystonia has been studied in detail earlier by Harting et al. (2009) and Garbade et al. (2014). These authors clearly showed that the severity of striatal lesions on MRI did correlate with the severity of the movement disorder. In contrast, extrastriatal MRI finding did not correlate with the motor dysfunction. In our study, 5/17 patients did not develop dystonia at a mean age of 37 months (3–132 months). Two of these patients (F10a and

Table 2 Mutations detected in 17 Indian patients with glutaric aciduria type 1

Case no.	Exon	Mutation at nucleotide level	Mutation at protein level	Frequency (alleles)	Type of mutations	Reported/ novel	PolyPhen2/SIFT prediction	References
F11a F11b	4	c.281G>A	p.Arg94Gln	2/34	Missense	Novel	Probably damaging/ damaging	This study
F10a F10b	6	c.401A>G	p.Asp134Gly	4/34	Missense	Novel	Probably damaging/ damaging	This study
F12	8	c.650C>T	p.Pro217Leu	1/34	Missense	Reported	–	Zschocke et al. (2000)
F11a F11b	8	c.662T>C	p.Leu221Pro	2/34	Missense	Novel	Probably damaging / damaging	This study
F3	8	c.764C>T	p.Ser255Leu	1/34	Missense	Reported	–	Busquets et al. (2000a)
F8	8	c.769C>T	p.Arg257Trp	2/34	Missense	Reported	–	Schwartz et al. (1998)
F9	8	c.770G>A	p.Arg257Gln	2/34	Missense	Reported	–	Schwartz et al. (1998)
F3	9	c.881G>C	p.Arg294Pro	1/34	Missense	Novel	Probably damaging/ damaging	This study
F15	9	c.937C>T	p.Arg313Trp	1/34	Missense	Reported	–	Goodman et al. (1998)
F15	11	c.1173delG	p.Asn392Metfs*9	1/34	Frameshift	Reported	Truncated protein	Anikster et al. (1996)
F12	11	c.1173dupG	p.Asn392Glufs*5	1/34	Frameshift	Novel	Truncated protein	This study
F4, F5, F6, F7	11	c.1204C>T	p.Arg402Trp	8/34	Missense	Reported	–	Biery et al. (1996)
F13, F14	11	c.1207C>T	p.His403Tyr	4/34	Missense	Reported	–	Wang et al. (2013)
F1	11	c.1238A>G	p.Tyr413Cys	2/34	Missense	Novel	Probably damaging/ damaging	This study
F2	11	c.1241A>C	p.Glu414Ala	2/34	Missense	Novel	Probably damaging/ damaging	This study

F12) had only extrastriatal features, whereas F2, F4 and F15 had both extrastriatal and striatal involvement. In our study, we did not perform any follow-up MRI. It has been shown (Heringer et al. 2010) that prompt vigorous intervention during acute episodes with the provision of glucose, fluids and electrolytes along with carnitine supplementation can prevent striatal degeneration and has improved outcome. Most of our patients had acute crises prior to the diagnosis. They were diagnosed after routine evaluation for either developmental delay, seizures or macrocephaly. Hence no emergency regimen was given at the time of acute precipitating factors. However, after initial diagnosis, patients were explained about emergency home treatment (Kolker et al. 2006, 2007, 2011).

An individualized long-term dietary treatment (Kolker et al. 2011; Viau et al. 2012; Lee et al. 2013) includes a low lysine diet through natural protein with or without lysine-free and tryptophan-reduced amino acid supplements along with L-carnitine supplementation for adequate growth (Boy et al. 2013). Due to lack of availability of lysine-free and tryptophan-reduced therapeutic formulae, the metabolic maintenance treatment was tried mainly through locally available protein-restricted diet with age-specific minimum requirement of lysine and tryptophan, micronutrients, riboflavin and carnitine supplementation. Baclofen was given for dystonia.

Mutation Spectrum

GA I mutations are classified either as severe or mild depending upon the residual enzyme activity and the urinary excretion of glutaric acid. p.Arg402Trp mutation in exon 11 is described as one of the severe and common mutations that account for about 20% of the mutations in Caucasians. Most of the patients are reported as compound heterozygotes, but in our cohort, 80% of the family mutations were in homozygous state including Arg402Trp mutation.

Figure 1 shows the distribution of 15 different mutations in five different exons of *GCDH* gene, in which seven of them (c.1238A>G, c.1241A>C, c.1173dupG, c.881G>C, c.401A>G, c.662T>C, c.281G>A) were novel and found in seven unrelated patients. Three families were homozygous for novel mutations, whereas five were compound heterozygous. Mutation prediction software PolyPhen2 and SIFT (Table 2) depicted that these missense mutations are probably damaging/damaging the protein. Frameshift mutations c.1173dupG (p.Asn392Glufs*5) and c.1173delG (p.Asn392Metfs*9) were generated truncated proteins. Family 11 had two novel mutations in compound heterozygous state, the father being a carrier of c.281G>A (p.Arg94Gln) and the mother a carrier of c.662T>C (p.Leu221Pro). However, these mutations appear as leaky or mild as both these sibs did not show urinary excretion of glutaric acid.

In Silico Analysis of Effect of Mutation on Their Structure

GCDH is a member of acyl-CoA dehydrogenase family and catalyses the oxidative decarboxylation of glutaryl-CoA using flavoprotein as its electron acceptor. Structural analysis reveals that the proper positioning of cofactor FAD (flavin adenine dinucleotide) and glutaryl-CoA is essential for decarboxylation reaction followed by dehydrogenation (Fu et al. 2004). Sequence analysis shows that residues Arg294, Tyr413 and Glu414 are highly conserved amongst the dehydrogenase family. Residue Glu414 is an active-site residue and functions as catalytic base. It is responsible for proton abstraction. Hence, the position of carboxylate group of Glu414 is extremely important and is stabilized by two hydrogen bonds with side chain of Arg294 (Fig. 2a). Therefore, Glu414Ala mutation will abolish the catalytic function of proton abstraction. The natural variant of Glu414 leads to loss of its enzymatic activity (Keyser et al. 2008). In addition, replacement of Glu414 by Ala also leads to a change in side chain conformation of crucial residues Arg294 and Tyr413 (Fig. 2b). Hence, this substitution will hamper its function severely. It clearly indicates that this mutation has a direct and crucial role in the pathogenesis of GA I.

Tyr413 is essential for the stabilization of FAD position through π–π aromatic interaction with phenyl ring of flavin (Fig. 2a). It is present on the flexible loop region, and conformation as well as the position of its aromatic side chain is stabilized by hydrogen-bonded interactions with another crucial residue Arg138. Arg138 plays an important role in enzymatic function as its replacement by Gln or Gly leads to manyfold increase in K_m value for glutaryl-CoA and decrease in its catalytic activity (Dwyer et al. 2001). The conformation of Arg138 side chain is known to be crucial for the enzymatic function as it acts as a hydrogen bond donor to γ-carboxylate of substrate glutaryl-CoA and electrostatic catalyst to stabilize the negative charges during catalysis (Keyser et al. 2008). Positively charged guanidinium group of Arg138 in wild-type model complex was in close proximity (3.0 Å) of carboxylate moiety of glutaryl-CoA. Substitution of Tyr413 by Cys will lead to loss of a crucial edge to face π–π interaction between Tyr and flavin; this might lead to destabilization of FAD position (Fig. 2c). Simultaneously this also alters the conformation of Arg138 as it is stabilized by hydrogen bond with Tyr413, and important guanidinium group in mutant model complex moves away from carboxylate group of glutaryl-CoA. Therefore, Tyr413Cys mutation causes the double adverse

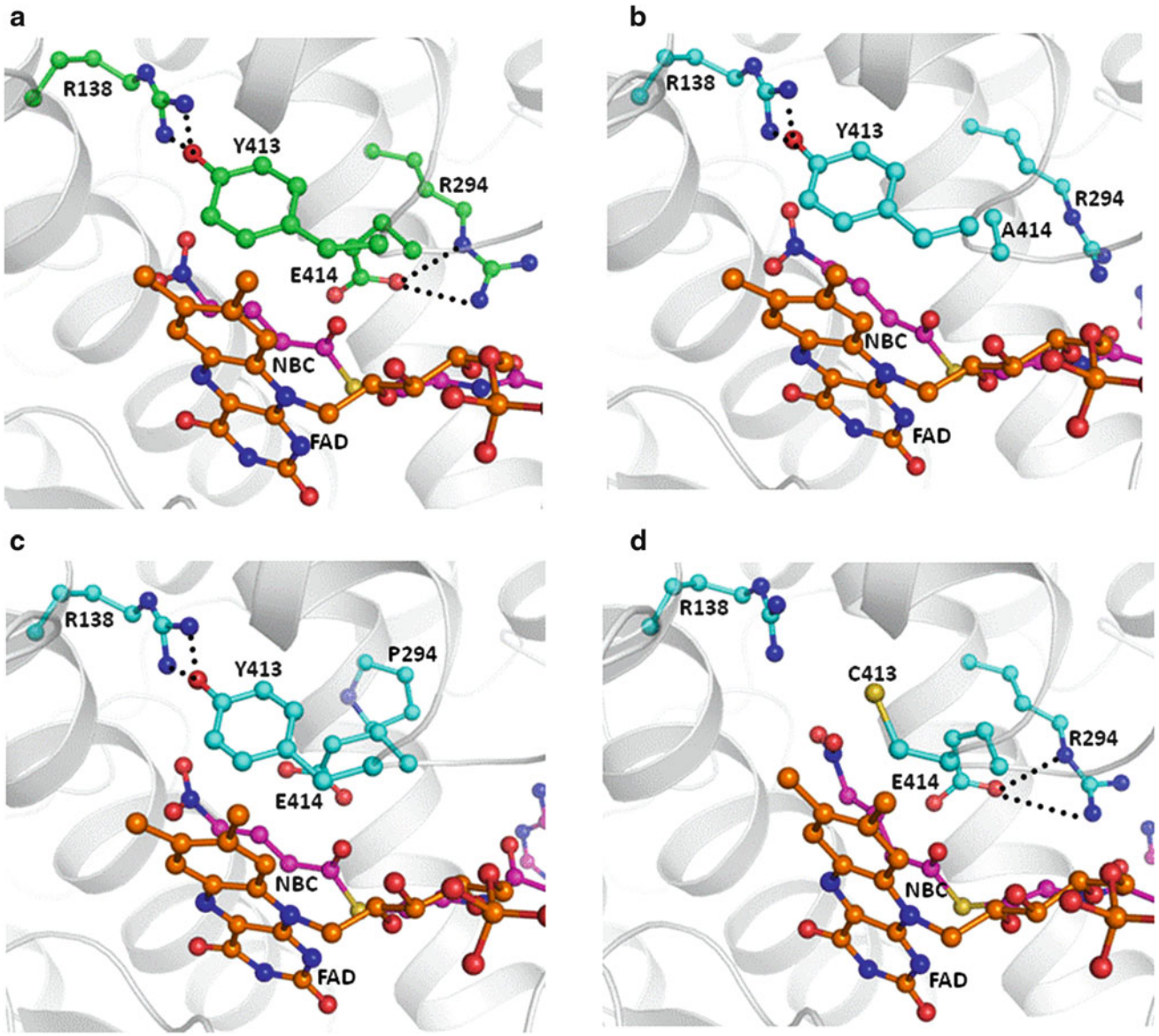

Fig. 2 Cartoon representation of (**a**) crystal structure of wild-type human mitochondrial GCDH and model structure of mutants (**b**) Glu414Ala, (**c**) Tyr413Cys and (**d**) Arg294Pro. Side chain of important residues are shown (ball and stick in *green* for wild type and *cyan* for mutants), glutaryl-Co-A (ball and stick in *magenta*) and cofactor FAD (ball and stick in *orange*) and hydrogen-bonded interactions in *black dotted lines*. Glutaryl-CoA and FAD are shown partially

impact and might lead to a significant decrease in the enzymatic activity of GCDH.

The substitution of Arg294 by Pro causes loss of crucial hydrogen bonds with Glu414, and this leads to a change in the conformation of active-site residue Glu414 required for proton abstraction (Fig. 2d). This also changes the loop conformation on which another crucial residue Tyr413 resides. Moreover, residue 294 lies in the helical region, and Pro is known to destabilize the helical conformation. Hence, this mutation might also adversely affect its enzymatic activity.

The rest of the three novel missense mutations (Arg94Gln, Asp134Gly and Leu221Pro) lie away from the active site. Residue Arg94 is positively charged and surface exposed. In mutant, Arg94 is replaced by Gln which is neutral in nature. This change in nature of residue can affect the interactions with the interacting partner. The degree of effect of this mutation on its function will depend on the importance of the interacting partner. Recently two important proteins, dihydrolipoamide *S*-succinyltransferase involved in glutaryl-CoA synthesis and β subunit of electron transfer flavoprotein acting as electron acceptor, have been identified as directly binding partners of GCDH (Schmiesing et al. 2014). Another mutation site residue Leu221 lies on the loop region and away from the active site. It is nonpolar in nature and its side chain is buried.

Its substitution by Pro does not affect the conformation of the protein significantly. Hence, this mutation does not appear to affect the enzymatic function of GCDH. Similarly Asp134 is pointing away from the active site; however, OD1 and OD2 of its side chain form hydrogen bonds with residues present on the neighbouring helices and might be assisting in the proper arrangement of those helices. Its substitution by Gly which lacks any side chain will not be able to form hydrogen bonds with residues on the neighbouring helices and might be decreasing the stability of helical arrangement. Though the role of this mutation on the catalytic function cannot be directly linked, this may lead to decreased enzymatic activity.

Biochemical, Genotype and Phenotype Correlation

The genotype and phenotype correlation has been elusive in GA I; however, genotype often predicts the biochemical phenotype in GA I. Severe mutations tend to have no residual enzyme activity and have a typical urinary metabolite pattern. Milder mutations have significant residual enzyme activity, hence low or normal urinary excretion of glutaric acids. Some of the mutations correlate well with the excretion of glutaric acid and 3 hydroxyglutaric acids. Arg402Trp and Ala293Thr correspond to high excretor group (Busquets et al. 2000a), whereas Arg227Pro and Val400Met account for low excretor group (Christensen et al. 1997). In our study, out of seven novel alleles, two (c.662T>C (p.Leu221Pro) and c.281G>A (p.Arg94Gln)) in exons 8 and 4, respectively, were low excretor alleles, whereas c.1241A>C (p.Glu414Ala), c.1173dupG and c.1207C>T (p.His403Tyr) in exon 11 were high excretor alleles.

In accordance with previous studies, we also did not observe any genotype and phenotype correlation (Christensen et al. 2004; Mushimoto et al. 2011). Interestingly, in our cohort, Arg402Trp (allele frequency 8/34, 23.5%) mutation emerged as one of the common mutations in four unrelated Muslim families. Two of these families had consanguinity. Haplotype analysis could not be done on these families to examine the founder effect. This mutation constituted about 23.5% of all the mutant alleles followed by His403Tyr (alleles frequency 4/34, 11.76%). Both these mutations were seen in unrelated families and did not have a similar phenotypic effect, suggesting a poor genotype–phenotype correlation and interfamilial variability in GA I. Intrafamilial variability as previously reported by Gregersen et al. (1977) and Kolker et al. (2007) was also observed in one of our families; the siblings in F10 and F11 (F10 showed presence of isolated macrocephaly and seizure in one sib and severe dystonia with regression in another sib) with the same mutation and genetic background had different phenotypes, suggesting intrafamilial variability in GA I. This intrafamilial and interfamilial variability can probably be explained by the epigenetic factors or complex interactions at tissue or metabolic levels and the extent of neurological damage caused by the various precipitating factors (Wang et al. 2013). In our cohort, exon 11 (in 53%; 18/34 alleles) followed by exon 8 (in 23.5%; 8/34 alleles) appear to harbour most of the mutations (Fig. 1). Hence, we recommend that molecular testing for exon 11 should be done first followed by exon 8 in resource-constrained settings. The combined diagnostic yield for these two exons appears to be as high as 76.5%.

Although the clinical, radiological and biochemical analyses are helpful in arriving at the diagnosis, molecular confirmation of the diagnosis undoubtedly gives an opportunity to the family for prenatal diagnosis in the subsequent pregnancies. Prenatal diagnosis could be offered in one family, and the pregnancy was terminated as the foetus was found to be affected.

The limitations in our study include qualitative results of urinary organic acids, because of which the exact correlation between biochemical phenotype and genotype could not be established. Secondly, after initiating the modified indigenous diet, the effectiveness of the diet could not be evaluated as the subsequent follow-ups for growth, biochemical monitoring for various amino acids and repeated follow-up MRI scans were not available.

In India, due to lack of uniformly available newborn screening, the diagnosis of GA I is delayed. Other factors for delayed diagnosis and poor final outcome could be lack of awareness amongst physicians, distance to the appropriate medical facility, lack of home management and poor patient compliance due to illiteracy and socioeconomic status. Excellent outcomes after early diagnosis and early dietary intervention for favourable neurological outcome have led to the inclusion of GA I in newborn screening panel across various countries. In countries like India, where a national newborn screening program is yet to start, authors would like to recommend its inclusion in the expanded panel. Till that time it would be plausible to have a high index of suspicion for early diagnosis and timely dietary intervention and parent education to prevent further progress of neurological damage and birth of another affected child. Although no formal biochemical follow-up evaluation has been done for the low-cost, culturally acceptable and locally available Indian indigenous diets, it was observed that with good patient compliance, patients on these diets did not further deteriorated neurologically. Biochemical evaluation with lysine and tryptophan levels and evaluation of growth parameters of Indian patients with GA I on indigenous diet are warranted to examine the effectiveness of these diets before drawing any conclusions.

Conclusion

Recognition of modifiable inherited metabolic disorders such as GA I requires high index of suspicion and timely

referral for further management to an appropriate centre. Early diagnosis and prompt management for acute illness and initiation of indigenous diet and carnitine are likely to have a beneficial effect on the neurological outcome. Further studies and appropriate follow-up are required to evaluate the effectiveness of Indian indigenous diets in the growth and long-term outcome of patients with GA. In accordance with the previous reports, our results also showed that there is a lot of intrafamilial and interfamilial variability even with the same mutation and there is no genotype–phenotype correlation. Molecular confirmation of the diagnosis is particularly helpful in prenatal diagnosis in the next pregnancy. p.Arg402Trp mutation emerged as a common mutation in Muslim families. Exon 11 and exon 8 are two hot spot exons and should be analyzed first in Indian patients for molecular diagnosis. Confirmation by molecular diagnosis aids in providing genetic counselling and prenatal diagnosis in future pregnancy.

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Take-Home Message

Exons 11 and 8 of the *GCDH* gene seem to be the mutational hot spot regions in Indian patients with GA I.

Compliance with Ethics Guidelines

Conflict of Interest

Neerja Gupta, Pawan Kumar Singh, Manoj Kumar, Shivaram Shastri, Sheffali Gulati, Atin Kumar, Anuja Agarwala, Seema Kapoor, Mohandas Nair, Savita Sapra, Sudhisha Dubey, Ankur Singh, Punit Kaur and Madhulika Kabra declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

Details of the Contributions of Individual Authors

Planning: Neerja Gupta, Pawan Kumar Singh, Sudhisha Dubey, Madhulika Kabra

Conducting: Neerja Gupta, Pawan Kumar Singh, Shivaram Shastri, Sheffali, Gulati, Atin Kumar, Anuja Agarwala, Seema Kapoor, Ankur Singh, Mohandas Nair, Savita Sapra, Sudhisha Dubey, Manoj Kumar, Punit Kaur and Madhulika Kabra

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RESEARCH REPORT

A Retrospective Survey Studying the Impact of Fabry Disease on Pregnancy

Alexandrea Holmes • Dawn Laney

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Abstract Fabry disease (FD) is a lysosomal storage disorder resulting from a deficiency of lysosomal enzyme α-galactosidase A (α-gal A). Reduced or missing α-gal A enzyme results in the storage of globotriaosylceramide (GL3) and related glycosphingolipids in the cellular lysosomes throughout the body. The majority of GL3 buildup occurs in the body's vasculature resulting in narrowed blood vessels and an increased risk for strokes, transient ischemic attacks, and deep vein thrombosis. Theoretical concerns have been raised about increased pregnancy complications in women affected by FD as glycosphingolipid storage has been found in both maternal- and fetal-derived placental tissues. This retrospective study was conducted to better understand risks for women with FD during pregnancy. Survey questions included queries about prenatal medications, teratogenic exposures, prenatal testing, common pregnancy complications, Fabry symptoms during pregnancy, obstetrical history, and immediate neonatal history. In total, 41 affected women completed the survey. Results indicate several Fabry-related symptoms and features may worsen during pregnancy, including gastrointestinal symptoms, acroparesthesias, proteinuria, headaches, and postpartum depression. Although no life-threatening complications were reported, a statistically significant increased frequency of hypertension was observed when comparing data from this study to the general population ($p < 0.05$) and previous publications ($p < 0.001$). Limitations include sample size and recall bias. Though this survey sampling of women was small and required women to recall their past pregnancy experiences, the findings suggest that when pregnant, women with FD should be aware of potential worsening of FD symptoms and may benefit from consulting with a maternal-fetal medicine specialist.

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Introduction

Fabry disease (FD) is an X-linked lysosomal storage disorder with an estimated incidence in at least 1 in 10,000 women in the United States (Desnick et al. 2001a, b; Kampmann et al. 2002; Laney et al. 2013). FD is caused by a deficiency or lack of the lysosomal enzyme α-galactosidase A (α-gal A; EC 3.2.1.22). Reduced amounts of α-gal A enzyme result in the buildup of globotriaosylceramide (GL3) and associated glycosphingolipids in the lysosome of cells throughout the body. GL3 storage in the endothelial lining of blood vessels leads to narrowing and constriction of cardiac, renal, and central nervous system vessels over time causing progressive damage of renal and epithelial cells, myocardial cells, neuronal cells, endothelial, and smooth muscle cells (Eng et al. 2006; Laney et al. 2013).

It has been conclusively shown that women with FD are not simply asymptomatic carriers. Over 60% of female heterozygotes suffer significant burden of disease and reduced quality of life (Wang et al. 2007). Frequent symptoms include hypertension prior to onset of worsening renal disease, severe abdominal cramping, hypohidrosis, central nervous system involvement with premature stroke, and psychological issues (Whybra et al. 2001; Ries et al. 2003; Gupta et al. 2005; Deegan et al. 2006; Wang et al. 2007; Wilcox et al. 2008; Bouwman et al. 2012). As more

than half of women with FD suffer from disease symptoms, the importance of evaluating disease status during pregnancy has been stressed and requires further investigation.

Specific concerns associated with females with FD during pregnancy include microvascular disease that could increase the risk for clotting and worsening kidney function (MacDermot et al. 2001; Eng et al. 2006). GL3 storage has also been documented in maternal- and fetal-derived placental tissues, which heightens the risk of constriction of the placental blood vessels (Vedder et al. 2006; Bouwman et al. 2010; Politei and Thurberg 2012). Additionally, worries have been raised about the status of an affected fetus causing pregnancy complications due to abnormal GL3 storage (Politei and Thurberg 2012). There are concomitant conditions that, when combined with preexisting FD, may complicate pregnancy. These include preeclampsia, gestational diabetes, hypertension, and maternal age at delivery (Macdonald-Wallis et al. 2011). Small-scale studies have shown that traditional FD symptoms such as acroparesthesias may worsen during pregnancy; however, no conclusive evidence of worsening of symptoms during pregnancy was seen in a previous large-scale review (Bouwman et al. 2012). The purpose of this retrospective survey is to increase our understanding of the actual risks in the previous pregnancies of women affected by FD as compared to reported rates of complications in the general population. Study objectives addressed this by determining the incidence of proteinuria onset and the rate of adverse events including transient ischemic attacks (TIAs), strokes, and deep vein thrombosis (DVTs) in pregnant females with FD as compared to pregnant women in the general population.

Methods

Institutional Review Board Approval

This project has been approved by the Emory University Institutional Review Board. All participants completed a detailed consent process prior to participating in the study. Documents approved for this study included recruitment flyers, recruitment emails, consent form, survey, and medical release form.

Participants and Recruitment

Recruitment for the study was a multifaceted effort. Survey recruitment flyers were mailed out to patients within the Emory University mailing lists who met inclusion criteria. Notice of this study was also included in the Fabry Support and Information Group (FSIG) monthly newsletter and on the National Fabry Disease Foundation (NFDF) website. The study investigators attended local and regional Fabry meetings sponsored by the NFDF and FSIG. Recruitment flyers were also distributed by genetic counselors working in Lysosomal Centers of Excellence around the United States to provide to qualified patients who may have an interest in participating. Participating institutions included Children's Hospital of Pittsburgh, Children's Hospital of Wisconsin, Cincinnati Children's Hospital, and Massachusetts General Hospital.

Study Population

After receiving IRB approval, participants who completed the informed consent process in person or via the telephone were screened to determine if they met study entry criteria. Inclusion criteria for participating in the IFOP study required all participants be female, over the age of 18 years, have a confirmed GLA gene mutation or low α-Gal A level on plasma leukocyte analysis, and have had a pregnancy within the past 25 years. Subjects not meeting these criteria were excluded. The target participant population was 100 pregnancies in at least 75 women.

Survey Design

In addition to providing consent, participants were asked to complete a medical release of information form in order to obtain medical records from the patient's obstetrician, gynecologist, and birth hospital. Study participants also completed a self-response survey entitled "The Impact of Fabry Disease on Pregnancy" (IFOP) about their pregnancies. The IFOP questionnaire was divided into five parts: demographic background, gynecological history, fertility, pregnancy history, and postnatal issues (Table 1). A sampling of patient survey questions are provided in Table 1. In total, the survey included at least 76 questions. Women were asked to complete the survey for each individual pregnancy, and questions were repeated for each subsequent pregnancy.

Statistical Analysis

Statistical support was provided by Emory University Biostatistics Center. All raw data was obtained from self-response queries and verified in the patient's medical record, if available. Data was then analyzed using comparative t-tests. Survey responses were totaled and compared to general population estimates of pregnancy complications from the Centers for Disease Control and Prevention Women's Health Survey (Centers for Disease Control and Prevention National Center for Health Statistics 2012) and

Table 1 IFOP survey sections and sample questions

IFOP survey section	Sample question asked
Pregnancy and gynecological history	1. "Have you ever been pregnant?" If yes, number of times.
	2. "At what age did you begin having your periods?"
	3. "Have you ever been evaluated for infertility concerns?"
Family history and pregnancy history	1. "Have you ever had a miscarriage?"
	2. "How many children have you given birth to?"
	3. "Did you know that you had Fabry disease before having children?"
Individual pregnancy history	1. "Did you smoke during your pregnancy?" If yes, how many cigarettes per day?
	2. "Did you have any of the following:"
	Gestational diabetes
	Abnormal ultrasounds
	High blood pressure
	Preeclampsia
	3. "Did you experience any of the following during pregnancy:"
	Increased diarrhea
	Constipation
	Alternating diarrhea and constipation
	4. "Did you have any heart problems during pregnancy:"
	Heart palpitations
	Atrial fibrillations
	Other heart problems
	5. "Did your doctor tell you had protein in your urine?"
Post-birth	1. "Was your baby born with any birth defects such as a heart defect or an extra toe?"
	2. Did you experience any symptoms of postpartum depression or anxiety?

Questions were presented to be answered as self-response multiple choice, yes or no, and free-response answers

previously reported literature values in woman affected by Fabry disease by using paired comparison tests. All statistical analyses were performed using SAS® software.

Results

Over 100 surveys were distributed to women affected by FD who met the inclusion criteria for the IFOP study. A total of 45 women were consented to participate, and ultimately, 41 women completed the survey and provided a medical records release for a response rate of approximately 45%. Records were obtained from physicians and hospitals for 21 out of the 41 participants. From the records, the investigators extracted clinic notes, laboratory values, and postnatal discharge summaries. The average age of the women at the time the surveys were completed was 43.5 years of age, and the average number of pregnancies per woman was 2.49. Responses collected from the survey are shown in Table 2.

Table 2 Demographic information of participants in the IFOP study

Demographic background	IFOP Participants
Ethnicity	34 Caucasian
	3 African Americans
	3 Hispanic
	1 Asian
Average age range of first pregnancy	11 women (12–21 years)
	21 women (21–29 years)
	8 women (30–35 years)
	1 woman (36–40 years)
Average number of pregnancies per participant	2.49 pregnancies
Average age of participants at time survey was completed	43.5 years

The most commonly reported FD symptoms and features experienced during the 100 pregnancies included proteinuria (37.20%), acroparesthesias (31.3%), headaches (22.5%), constipation (29.40%), and diarrhea (27.5%) (Table 3). No life-threatening complications such as renal failure, stroke, TIAs, or DVTs were reported during

Table 3 Most common symptoms and laboratory abnormalities of Fabry disease experienced during pregnancy by affected women

Most common Fabry symptoms during pregnancy	Number of pregnancies with increased symptoms	Total number of pregnancies	Incidence (%)
Proteinuria[a]	38	102	37.20
Acroparesthesia	32	102	31.30
Headaches	23	102	22.50
Constipation	30	102	29.40
Diarrhea	28	102	27.50

[a] Proteinuria was not formally quantified

Table 4 Comparison of pregnancy complications in affected women with FD during pregnancy and the general population of pregnant women

Pregnancy complication	Incidence this cohort of pregnancies	Literature values in Fabry patients from Bouwman et al. (2012)[a] and Wang et al. (2007)[b]	*P* value, comparing to literature	General population incidence	P value, comparing to general population
Preeclampsia	4.9% ($n = 5/102$)	9.4% ($n = 3/32$)[a]	0.35[a]	3.4%	0.40
Proteinuria	37.2% ($n = 38/102$)	34% ($n = 11/32$)[a] 56% ($n = 10/18$)[b]	0.76[a] 0.14[b]	3.8%	$<2.2 \times 10^{-16}$
Gestational diabetes	8.8% (9/102)	–	–	2–10%	0.002–0.86
Premature delivery	18.8% (16/85)	19% ($n = 6/32$)[a]	0.99[a]	11.9%	0.06
Hypertension	10.8% (12/102)	34% ($n = 11/32$)[a] 43% ($n = 16/37$)[b]	0.001[a] 1.91×10^{-5} [b]	6%	0.05
Miscarriage	11.8% (11/102)	25% ($n = 8/32$)[a]	0.06[a]	9–12%	0.30
Intrauterine death	2.3% (2/85)[c]	0.03% ($n = 1/32$)[a]	0.81[a]	0.625%	0.09

Symbols denote literature comparisons, Bouwman et al. (2012)[a] and Wang et al. (2007)[b]. Population incidence of complications is from the CDC (Centers for Disease Control and Prevention National Center for Health Statistics 2012)
[c] These pregnancies were affected by chromosomal abnormalities

pregnancy. Of the symptoms reported, proteinuria was reported most often. Of the 41 women who completed the survey, 4 were treated with enzyme replacement therapy (ERT). No complications of ERT were reported.

Pregnancy complications queried in the survey included preeclampsia, proteinuria, gestational diabetes, premature delivery, pregnancy-induced hypertension, miscarriage, and intrauterine death. As seen in Table 4, the incidence for preeclampsia, gestational diabetes, premature delivery, and miscarriage as compared to the general population rate was not statistically significant. The rate for intrauterine death was significant; however, both babies were affected by chromosomal abnormalities and as such do not reflect an increased incidence of intrauterine death related to FD.

The rate of proteinuria and pregnancy-related hypertension in reviewed FD cases was statistically significant and presents at a higher rate as compared to the general population rate. Protein levels were increased in 38/102 (37.2%) pregnancies. Analyzing the data for proteinuria onset by subject rather than pregnancy, 17 women out of 41 women affected by FD were found to have proteinuria during at least one pregnancy (Table 5). Within this cohort, 7/17 women had new onset of proteinuria in their initial pregnancy with an additional 3/17 women experiencing proteinuria onset not in their first pregnancy but in a subsequent pregnancy.

Pregnancy-related hypertension was reported in 10.8% (12/102) of the pregnancies. Some of these pregnancies 4.9% (5/102) were also complicated by preeclampsia. However, none of the six women reporting pregnancy-related hypertension were affected by hypertension prior to their first pregnancy.

Although few complications were experienced in the postpartum period, we did find that postpartum depression was reported in a surprising 17.1% of this cohort of women (Table 6). This value is not statistically significant as compared to the general population incidence, but it did affect a significant number of participants (7/41). Of the

Table 5 Individual participant responses to pregnancy complications (proteinuria, hypertension, and depression/anxiety)

Women with significant pregnancy complications					
Pregnancy complication	Symptoms before pregnancy	First pregnancy	Affected pregnancies per participant	Total number of women reporting complications ($n = 41$)	Affected pregnancies ($n = 102$)
Proteinuria	Yes	Yes	2/2	17/41	38/102
	No	No	1/2		
	No	Yes	2/2		
	No	Yes	2/4		
	Yes	Yes	2/2		
	No	Yes	1/2		
	No	Yes	2/2		
	Yes	Yes	2/3		
	No	No	2/4		
	No	Yes	4/4		
	Yes	No	2/2		
	Yes	Yes	2/2		
	No	No	3/4		
	No	No	2/4		
	No	Yes	2/2		
	Yes	Yes	2/4		
	Yes	Yes	5/5		
Hypertension	No	No	2/2	6/41	11/102
	No	Yes	2/2		
	No	Yes	1/1		
	No	Yes	2/2		
	No	Yes	2/2		
	No	Yes	2/4		
Depression/ anxiety	No	Yes	1/1	7/41	10/102
	No	No	1/2		
	No	Yes	2/2		
	No	No	1/2		
	Yes	Yes	1/2		
	No	Yes	1/2		
	No	Yes	3/5		

This table shows reported responses for women prior to pregnancy, first pregnancy, and total pregnancies

seven women who reported postpartum depression, only one participant reported having a prior history of depression and anxiety (Table 5).

Discussion

Retrospective investigation into pregnancies in women affected by FD found an increased prevalence of several specific pregnancy complications as compared to the general population of pregnant women. Results indicate several FD symptoms may worsen during pregnancy, including gastrointestinal symptoms, acroparesthesias, proteinuria, headaches, and depression during the postpartum period. This study also confirms that females with FD report a significant incidence of proteinuria and hypertension during pregnancy; however, there was no increased incidence of strokes, end-stage renal disease, or other related life-threatening complications in this population. Building on the current FD guidelines, this survey supports the recommendation that pregnant women with FD be evaluated and, in some cases, monitored throughout pregnancy by a maternal-fetal specialist in addition to standard prenatal care (Laney et al. 2013).

The survey results can assist providers caring for women with FD during pregnancy. Although progression of renal

Table 6 Incidence of postpartum depression in women with Fabry disease

Women reporting postpartum depression/ anxiety	General population postpartum depression incidence	Literature, Bouwman et al. (2012)	*P* value
17.1% ($n = 7/41$)	9.1% (CDC in 2009)	55% ($n = 34/62$)[a]	0.07
Stratifying based on age:			
<30 years $n = 5$			0.19
>30 years $n = 2$			0.11

[a] This study did not investigate incidence of depression postpartum but in general in females with FD

disease could not be determined from the dipstick analysis data in our study, urinary protein levels should be monitored in women with FD throughout the entire duration of their pregnancy and addressed using established clinical guidelines (Laney et al. 2013). The reported incidence of hypertension in the pregnancies of participants compared with literature and the general population also suggests that women with FD should have their blood pressure monitored when pregnant and should also be recognized that unlike in the general population of pregnant patients, the increased rate of pregnancy-related hypertension in our study seems unrelated to an increase in preeclampsia or preexisting obesity.

Although not found to be a statistically significant risk as compared to the general population rate, signs and symptoms of postpartum depression should still be monitored in prenatal and postpartum period. The risk for depression and anxiety in women affected by FD is increased and is experienced by half of affected women, even when they are not postpartum (Wang et al. 2007; Bouwman et al. 2012; Laney et al. 2013; Bolsover et al. 2014).

The increased Fabry-related symptoms such as acroparesthesias in hands and feet, headaches, constipation, proteinuria, and diarrhea should be monitored and treated in pregnancy to optimize patient comfort and prevent disease progression. Of course, these women may require a change in therapeutic regime and consultation with a genetic counselor or teratogen service as some medications used to treat symptoms may have a teratogenic effect.

The use of ERT during pregnancy to treat the root enzyme deficiency in FD has only been evaluated in limited studies. Accordingly, the decision to start or continue therapy must be made on an individual basis in cooperation with a team experienced with treatment of FD (Deegan et al. 2006; Vedder et al. 2006; Parent et al. 2010). This is further supported by established recommendations from the National Society of Genetic Counselors which recommends that women with FD be placed on ERT as soon as symptoms are first experienced and use discretion on whether to continue during pregnancy (Laney et al. 2013). In this study, 4 out of 41 women received ERT infusions throughout the duration of the pregnancy.

There are several limitations to this study. The use of a small sample size may have obscured statistical significance that may be discovered in larger studies. Also, the questionnaire used was created by the investigators and has not been formally validated. The retrospective nature of the study might have led to recall bias although use of medical records to validate findings sought to avoid this issue. In future studies, the investigators will likely change the inclusion criteria to require a more recent pregnancy or prospective enrollment to address the difficulties in obtaining medical records on all patients. Although medical records were requested to allow for verification of participant self-responses, medical records were not received for all study participants. Data analysis may be a limitation as the few studies regarding pregnancy complications in FD all differ in methods of data collection and sample size. This may limit the ability to compare these studies. Data stratification based on race or ethnicity was not included as the majority of participants were Caucasian. We acknowledge that this analysis may overrepresent the complications reported due to the fact that multiple pregnancies were analyzed from the same woman. The authors chose to analyze data collected based on number of pregnancies due to the many uneventful pregnancies reported. Additionally, the participants who had problematic pregnancies may have been more eager and willing to complete the survey thus creating participation bias.

Results from this survey highlight a need for future studies to inquire about complications and health concerns of pregnant women affected with FD. Studying the quality of life reported by women during pregnancy would assist in determining how disease may affect pregnancy. In the future, a multicenter prospective analysis of pregnant women with FD may aid in providing more concrete information about the specific risks of complications during pregnancy.

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who participated in completing the IFOP survey. We hope the data collected from this survey will further benefit women with Fabry disease.

Synopsis

Based on retrospective review, pregnant women affected by Fabry disease do not have life-threatening complications but may experience worsening of specific disease symptoms.

Compliance with Ethics Guidelines

Alexandrea Holmes declares no conflicts of interest.
Dawn Laney has received research grant support from Genzyme Corp., Amicus Therapeutics, Synageva Corporation, and Shire Plc and serves on the Genzyme Fabry Registry Board.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentations (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

Contributions

Planning of this study and oversight was coordinated by Dawn Laney.
Data collection, analysis, and preparation of this manuscript were performed by Alexandrea Holmes.

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RESEARCH REPORT

Evaluation of Implementation, Adaptation and Use of the Recently Proposed Urea Cycle Disorders Guidelines

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Abstract *Background*: Implementation of guidelines and assessment of their adaptation is not an extensively investigated process in the field of rare diseases. However, whether targeted recipients are reached and willing and able to follow the recommendations has significant impact on the efficacy of guidelines. In 2012, a guideline for the management of urea cycle disorders (UCDs) has been published. We evaluate the efficacy of implementation, adaptation, and use of the UCD guidelines by applying different strategies.

Methods: (i) Download statistics from online sources were recorded. (ii) Facilities relevant for the implementation of the guidelines were assessed in pediatric units in Germany and Austria. (iii) The guidelines were evaluated by targeted recipients using the AGREE instrument. (iv) A regional networking-based implementation process was evaluated.

Results: (i) Download statistics revealed high access with an increase in downloads over time. (ii) In 18% of hospitals ammonia testing was not available 24/7, and emergency drugs were often not available. (iii) Recipient criticism expressed in the AGREE instrument focused on incomplete inclusion of patients' perspectives. (iv) The implementation process improved the availability of ammonia measurements and access to emergency medication, patient care processes, and cooperation between nonspecialists and specialists.

Conclusion: Interest in the UCD guidelines is high and sustained, but more precise targeting of the guidelines is advisable. Surprisingly, many hospitals do not possess all facilities necessary to apply the guidelines. Regional network and awareness campaigns result in the improvement of both facilities and knowledge.

Communicated by: Jerry Vockley, M.D., Ph.D.

Competing interests: None declared

On behalf of the working group for the "European guidelines for the diagnosis and management of urea cycle disorders," further members of this working group are Nathalie Boddaert, Alberto Burlina, Anupam Chakrapani, Carlo Dionisi-Vici, Marjorie Dixon, Daniela Karall, Martin Lindner, Diego Martinelli, Vicente Rubio, Pablo Sanjurjo Crespo, René Santer, Aude Servais, and Vassili Valayannopoulos.

Introduction

Medical decisions should ideally be based on firm data obtained from well-designed studies. However, many fields in medicine lack such information – especially when rare diseases are involved – rendering the patient's management subject to individual doctors' best knowledge and experience as well as to the advice of limited numbers of experts (Arnold et al. 2008, 2009; Spiekerkoetter et al. 2009). In an attempt to overcome such "eminence-based" approaches, guideline projects have been initiated to collect, carefully discuss, and weigh all available information on the basis of standardized methodological strategies and finally propose the highly needed recommendations and guidance for medical decision making (Kishnani et al. 2010; Kölker et al. 2011a). There has been some criticism addressing the standardized methodological strategies applied because those have been developed to evaluate large pharmacological trials but not research in the field of rare diseases (Vockley et al. 2013). While this criticism is appropriate for the SIGN methodology (Scottish Intercollegiate Guideline

Network, http://www.sign.ac.uk), the recently proposed GRADE (short for Grading of Recommendations Assessment, Development and Evaluation) approach aims at ameliorating this shortcoming (http://www.gradeworkinggroup.org/).

The list of guidelines is currently steadily growing, and according to the intention of such processes, this should result in some benefit for the patients. First experiences in the field of rare inborn errors of metabolism (IEM), e.g., with the guidelines addressing glutaric aciduria type 1, support this assumption (Heringer et al. 2010). We have recently proposed a guideline for the management of urea cycle disorders (UCDs) (Häberle et al. 2012), a group of rare defects of ammonia detoxification. The process of this guideline development involved the SIGN methodology as of 2009 and a standardized nominal group discussion approach. Target groups for the guidelines were metabolic specialists and nonspecialists in the metabolic field. Aims of the guidelines encompassed harmonization of diagnostic, therapeutic, and disease monitoring strategies in expert centers as well as raising awareness for the disease to enhance the probability for early diagnosis and effective treatment in the nonexpert field.

Targeted implementation of guidelines and the assessment of their adaptation are a complex and not extensively investigated process in the field of rare diseases. This study investigates the general interest in the published guidelines and addresses the following questions: Do the guidelines reach the targeted readers? Do nonexperts consider understandable, reliable, and feasible in everyday practice what experts have written? Do users of the guidelines have the facilities and resources available to follow the recommendations? These are questions important to answer in order to tailor future updates of existing guidelines or in the planning of novel ones (Knai et al. 2012; Legido-Quigley et al. 2012).

Accordingly, we evaluated the efficacy of implementation, adaptation, and use of the UCD guidelines, and hereby the "real-world" impact, within a proportion of the target population by applying different strategies. The aim of this publication is not only to document the current situation with respect to the UCD guidelines, but in addition to motivate colleagues to consider the evaluation and implementation of any planned guidelines already during their development.

Methods

Download Statistics from Online Sources

Implementation strategies included publication of the full guideline text as well as the method report from July 2012 on the website of the "Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften" (AWMF; http://www.awmf.org/leitlinien/detail/ll/027-006.html), the well-established German guideline server. The AWMF follows a strict protocol to determine the classification of guidelines. Downloads from this source are free of charge. Generally, the AWMF guidelines are also accessed from German-speaking physicians from Switzerland and Austria.

Additionally but with a different format and scope, the UCD guidelines were published in an open access online journal specially addressing rare diseases, the Orphanet Journal of Rare Diseases (OJRD; http://www.ojrd.com/content/7/1/32 (Häberle et al. 2012)). To evaluate the guidelines' quantitative use, the frequency of downloads from both online sources since publication at the AWMF server on July 9, 2012 until May 31, 2014 (data missing for months January and February 2014 due to technical problems; total 632 days) and since publication at the OJRD server on May 29, 2012, until May 21, 2014 (total 722 days) was counted.

Assessment of Facilities Necessary for the Practical Implementation of the Guidelines in Pediatric Units

To estimate whether resources in pediatric units were suitable to enable physicians to act on the basis of the guidelines' recommendations, a brief Internet-based survey (Survey Monkey; https://de.surveymonkey.com/) was sent out to all pediatric units in Germany ($n = 408$) and Austria ($n = 238$). The survey addressed the following issues:

- Hospital category: primary, secondary, or tertiary care center
- Availability of ammonia measurements: 24/7, only during main working hours, or in an external lab only
- Time span between collection of sample and result: <1 h, 1–3 h, 3–6 h, >6 h
- Mode of ammonia determination: bedside test, lab test
- Sample and preanalytical requirements (e.g., capillary or venous blood, cooling of sample)
- Availability of emergency drugs (nitrogen scavengers, L-arginine) in the hospital

Data presented are pooled from both countries.

Guideline Assessment Using the AGREE Instrument

For content and process evaluation of the guidelines, we randomly selected 46 German and 26 Austrian target users from an alphabetical list of the departments of pediatrics, neurology, and neuropediatrics (Germany only). Heads of departments were asked to complete the AGREE instrument (http://www.agreetrust.org/resource-centre/the-original-agree-instrument/) or to delegate this task to one of their coworkers. The AGREE instrument is a standardized

tool, developed in 2003 to "advance the science of guidelines," and has since then become the "international gold standard for practice guidelines evaluation and development" (Burgers et al. 2004).

Regional Implementation of Guidelines

Finally, we implemented the guidelines following a regional networking approach in Vorarlberg, the most western province of Austria. Due to the geographical structure of the area, medical care for patients from the region is offered mainly by regional medical services. The implementation strategy focused on the three pediatric departments in the region. The implementation process encompassed the following phases:

1. Stakeholder consent: setup of a first contact to the heads of the three pediatric departments to obtain consent and support for the implementation.
2. Awareness raising for UCDs and the guidelines: a short presentation of the main basic topics covered by the guidelines was given to the medical staff of the three departments.
3. Discussion of practical questions in every department, such as facilities for ammonia measurement, clinical symptoms which should prone immediate assessment of ammonia, availability of emergency medications, triage settings for (potentially) affected patients, etc.
4. Provision of assistance concerning practical issues (ordering of drugs, laboratory facilities, which forms to fill, how to get access to specialist advice, etc.).
5. Interactive elaboration of triage and decision trees for each department.

The following outcome parameters for the evaluation of the implementation strategy were measured before and after the intervention:

1. Availability of laboratory facilities
2. Number of ammonia measurements/department (mean per 6 months)
3. Availability of and knowledge about emergency drugs (interview medical staff)

Results

Download Statistics from Online Sources

Download numbers for both online available versions of the UCD guidelines are shown in Table 1.

Remarkably, there was an increase in downloading over the time indicating an ongoing and even growing interest in the guidelines (Fig. 1). However it was impossible by limitations of the method to differentiate between first and repeated downloads from single users.

Table 1 Numbers of downloads from OJRD and AWMF

Time	Orphanet Journal of Rare Diseases (OJRD)	AWMF server
Last 30 days	1,070	328
Last 365 days	10,896	2,882
Total	18,817	3,894
Total per day[a]	26.1	6.2

[a] Total days 722 and 632 for OJRD and AWMF, respectively

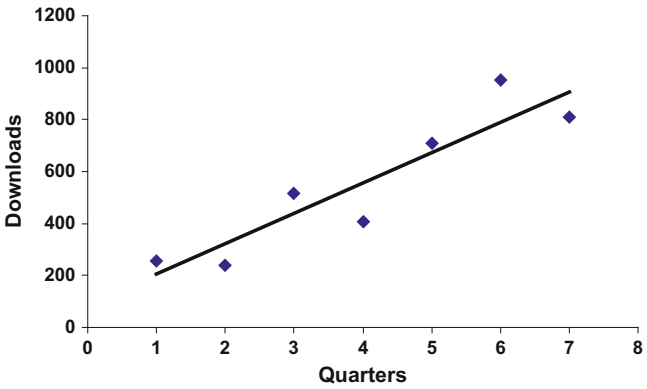

Fig. 1 Number of downloads per quarter from the German AWMF guideline server is shown for the period from July 2012 until May 2014

Assessment of Facilities Necessary for the Practical Implementation of the Guidelines in Pediatric Units

The overall response rate to the Internet-based survey was 10.4%; response rates were 14% in Germany (57 responses from 408 addressants) and 4.2% in Austria (10 responses from 238 addressants). Respondents represented primary care hospitals in 30%, secondary in 9%, tertiary in 46%, and tertiary at university in 15%. Detailed results are summarized in Table 2.

Remarkably, emergency drugs were not available in 12 of 31 tertiary nonuniversity hospitals (39%) and in 10 of 20 primary care hospitals (50%).

Guideline Assessment Using the AGREE Instrument

After sending out the request to complete the AGREE instrument to a total of 72 colleagues (46 in Germany, 26 in Austria), we did not receive any response from 64 addressants. Two colleagues returned the completed form without queries. Six addressants expressed that they considered themselves not competent to fill the survey

Table 2 Survey on resources in pediatric units (pooled data from the 67 responding hospitals)

Availability of ammonia measurements	**24/7**	**Only during main working hours**		**In an external lab only**		
	82%	9%		9%		
Time to result after sample collection	**<1 h**	**1–3 h**	**3–6 h**	**>6 h**		
	63%	27%	9%	1%		
Mode of ammonia determination	**Bedside test**	**Lab test**	**Lab test during the day but bedside test outside normal working hours**			
	1%	96%	3%			
Sample requirements[a]	**Capillary**		**Venous**			
	9%		94%			
Preanalytics	**Cooling of sample**	**Fast transport**	**Lab informed**	**Special tubes**	**Special sampling**	**Special sample transport**
	60%	88%	36%	25%	16%	9%
Availability of emergency drugs	**On the ward**	**Available within 1 h**		**Not available**		**Unknown**
	44%	13%		34%		9%

[a] Some labs accept both types of samples

because of not being an expert in the metabolic field. Nevertheless, two of these colleagues agreed to fill in the AGREE instrument after having being reassured that the status of being a metabolic expert was not conditionary.

The low number of only four completed surveys hampers systematic evaluation of the results but is in full agreement with the AGREE research group recommending "that each guideline is assessed by at least 2 appraisers and preferably 4" (http://www.agreetrust.org/about-the-agree-enterprise/introduction-to-agree-ii/preparing-to-use-the-agree-ii/). However, although being in agreement with the AGREE research group, we consider the small number of responses a limitation. Nevertheless, there were two points raised by all respondents: the views and wishes of the patients were said to be not ascertained to their full extent by the guideline developers (question 5; score 2 on a scale of 4 to 1 with 4 being best). In addition, costs that possibly result from use of the guidelines were supposedly not taken into account (question 20; score 2 on a scale of 4 to 1 with 4 being best).

Regional Implementation of Guidelines

In the region of Vorarlberg, 52 ammonia measurements had been carried out during 6 months before implementation of the guidelines in two of the three regional departments. In the first 6 months following the intervention, number of ammonia measurements increased to 106.

In the third department, ammonia measurement had not been available in-house but had to be sent to an external laboratory with a turnaround time of >24 h. To the memory of the medical staff, ammonia measurement had "very rarely to never" been ordered. Following the intervention, measurement of ammonia within 1 h time (24/7) has been established in the hospital lab.

Before the intervention, medical staff in all pediatric departments was not familiar with emergency drugs for the treatment of hyperammonemia, and access to the drugs was not regulated. In the context of the intervention, a list with drugs, details of drug preparation, and dosages to be used in hyperammonemic patients has been established on the basis of the guideline recommendations. Drugs have subsequently been stored in one hospital pharmacy and are available on request 24/7 with a maximal delay of two hours for all three departments. On all intermediate and intensive care units, in-house storage of basic emergency drugs has been instituted. With regard to triage and patient pathways, regional resources were discussed, and it was agreed that since the most effective extracorporeal detoxification technology was not available in the region, patients with persistent or progressive hyperammonemia must be referred to a tertiary institution in due time. In addition it was agreed that advice from a metabolic expert should be obtained as early as possible in the process, and contact details were made available for the clinicians.

In the 6 months before the intervention, one child with neonatal hyperammonemia had been diagnosed on day three after admission. Since implementation of the guidelines, one neonate with acute neonatal hyperammonemia has been identified. Ammonia measurement has been conducted within two hours after admission, and subsequently metabolic specialist advice has been obtained. Treatment has been initiated according to the guidelines, and the patient was transferred to a tertiary center due to progressive hyperammonemia.

Discussion

Guidelines are produced in the intention to guide medical decisions in patient care. Readers ideally expect positive effects of specific recommendations on clearly defined (outcome) parameters. In children with glutaric aciduria type 1, a significant benefit is seen when treatment during acute illness adheres to the guideline recommendations (Heringer et al. 2010). Such an evaluation requires detailed knowledge of the natural course of the disease and patients' perspectives and the formulation of relevant and meaningful outcome parameters. Examples of possible outcome parameters include the survival rate of patients with neonatal presentation, the frequency of hyperammonemic crises during the course, the delay in diagnosis, or the long-term intellectual outcome. Respective data are so far only randomly available for the UCD population. The lack of data underlines the importance of currently ongoing UCD registry projects in the USA, Europe, and Japan intending to pool data from large patient populations systematically (Kölker et al. 2011b; Tuchman et al. 2008).

On the basis of the frequency by which the UCD guideline paper was accessed and downloaded from the open access available online sources, the paper was and still is categorized as "highly accessed" in the *Orphanet Journal of Rare Diseases*. In the case of the AWMF guideline server, download numbers constantly increased during the first two years after publication (Fig. 1) indicating that the knowledge about the availability of the UCD guidelines was (and still is) growing. Nevertheless, given the rarity of UCDs, the strong and ongoing interest in the guidelines indicated by high and constant download numbers is remarkable. However, it must be kept in mind that repeated downloads from single users could not be differentiated from first downloads. In addition, we cannot infer from these data whether download resulted in any practical consequences. Likewise, it remains open whether readers belong to the expert or nonexpert groups.

The survey sent to pediatric hospitals revealed some remarkable results concerning the availability of ammonia measurements and emergency drugs as well as preanalytical standards. In only 82% of the hospitals, ammonia measurement can be performed at all times, while 9% of the hospitals offer this service only during working hours, and another 9% need to send the sample to an external lab. This seems to be a critical issue since a relevant proportion of acutely hyperammonemic patients will in these circumstances not receive timely analysis of plasma ammonia. This fact automatically results in a delay in diagnosis and treatment initiation. In addition, there seems to be wide variation of preanalytical procedures in use with cooling of the blood sample and requirement to beforehand inform the lab in 60% and 36% of institutions, respectively (Table 2). Another finding from this nonrepresentative survey is even more worrying: in 34% of the hospitals, emergency drugs (nitrogen scavengers and L-arginine) for the treatment of hyperammonemia are not available. Among the hospitals without in-house drug availability, 39% are tertiary nonuniversity hospitals and thus by definition dedicated to care for patients with complex, severe disorders such as UCD. It should be noted however that this survey does not represent a pre- versus post-guideline evaluation. We like to highlight this shortcoming mainly as a suggestion for future guideline groups when authors could (and should) perform a true pre- versus post-evaluation.

Implementation of guidelines into clinical practice resulting in the induction of behavioral change is challenging to achieve (Gross et al. 2001; Legido-Quigley et al. 2012). Publishing guidelines – even on open access platforms – is one of the least effective interventions in that respect (Gross et al. 2001). If enhancement of awareness toward the value of ammonia measurements in a variety of clinical conditions is the target, guidelines only available by searching actively for the term "hyperammonemia" will not be effective. Connecting the guideline to more clinical, symptom-oriented search terms (e.g., impaired consciousness, coma) might help to ameliorate this shortcoming in the future.

Presentation by expert talks likewise often fails to induce changes in clinical practice (Gross et al. 2001). Along this, a recent suggestion to improve surveillance for hyperammonemia by introducing "an electronic medical record-based tool to assist physicians in the detection of hyperammonemia" deserves attention (Vergano et al. 2013). The implementation of such warning systems may prove superior efficacy if compared to education-based strategies classically intended by expert talks.

Our experience in implementing the UCD guidelines in a regional medical system underlined the finding that implementation of guidelines by networking activities and interactive approaches is the most effective strategy to induce behavioral change in everyday practice. This can be attributed to the active involvement of target groups and to the potential to adapt methods to local resources or local resources to yet unmet medical needs as well as to the lowering of thresholds to seek expert advice (Gross et al. 2001).

Conclusion

The data obtained by our evaluation indicate that interest in the UCD guidelines is generally high and sustainable. Nevertheless, more precise targeting of the guidelines is advisable. In the future, registry data may develop into a sound basis for significant outcome parameters the guidelines attempt to change. With regard to implementation of guidelines for a group of rare diseases, our experience

suggests that a regional network and awareness campaign approach improve medical care. Sustainability of improved awareness, patient care, and outcome are parameters of interest for future research in this field.

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Compliance with Ethics Guidelines

Conflict of Interest

Johannes Häberle and Martina Huemer declare that they have no conflict of interest.

Animal Rights

This article does not contain any studies with human or animal subjects performed by the authors.

Contributions of Each Author

J. Häberle and M. Huemer have together planned and performed the conception, design, analysis, and interpretation of data and drafted the article and revised it.

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RESEARCH REPORT

Autophagy in Natural History and After ERT in Glycogenosis Type II

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Abstract We studied the role of autophagy in a series of 10 infantile-, juvenile-, and adult-onset GSDII patients and investigated autophagy blockade in successive biopsies of adult cases during disease natural history. We also correlated the autophagosome accumulation and efficiency of enzyme replacement therapy (ERT) in four treated cases (two infantile and two juvenile-adult onsets).

The autophagic flux was monitored by measuring the amount of p62-positive protein aggregates and compared, together with fibre vacuolisation, to fibre atrophy.

A blocked autophagic flux resulted in p62 accumulation, increased vacuolisation, and progressive atrophy of muscle fibres in biopsies collected from patients during natural history. On the contrary, in the GSDII cases early treated with ERT, the autophagic flux improved and muscle fibre atrophy, fibre vacuolisation, and acid phosphatase activity decreased.

The functionality of the autophagy-lysosome system is essential in GSDII muscle, which is characterised by the presence of swollen glycogen-filled lysosomes and autophagic build-up. Defining the role of autophagy and its relationship with muscle loss is critical for understanding the disease pathogenesis, for developing new therapies, and for improving ERT efficacy in GSDII.

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Introduction

Glycogenosis type II (GSDII, MIM# 232300) is an autosomal-recessive disorder caused by the deficiency of the lysosomal enzyme acid α-glucosidase (GAA), which catalyses the hydrolysis of α-1,4 and α-1,6 links of glycogen (Angelini and Engel 1973; Hirschhorn and Reuser 2001; van der Ploeg and Reuser 2008). The enzyme deficiency leads to a spectrum of clinical phenotypes ranging from an infantile and rapidly fatal form (Pompe disease) to a slowly progressive late-onset form (Laforet et al. 2000). The slow progression of the disease and the variable organ involvement complicate the prognosis and the efficacy of therapy of adult GSDII cases. Untreated adult GSDII patients have a poor quality of life (Hagemans et al. 2004).

The first successful trials with enzyme replacement therapy (ERT) were done on selected infantile Pompe patients, because of the severity of the disease and the rather homogeneous natural course (Kishnani et al. 2007). Recombinant human α-glucosidase (rhGAA) treatment was demonstrated to be effective in reducing left ventricular mass and improving survival in infantile patients. In adults, treatment efficacy is variable (Angelini et al. 2012). We aim to describe morphological changes and autophagy biomarkers in muscle biopsies from both infantile and adult forms of GSDII.

Material and Methods

Patients

We selected ten patients (three Pompe, seven adult onset) with molecular diagnosis of GSDII (Table 1), who underwent clinical examination, muscle biopsy, measurement of

Table 1 Clinical and genetic data of GSDII patients

Pt. No.	Biop. No.	Phenotype	ERT duration	Onset	Age at biopsy	Disease duration	Ventilator use	GAA gene mutations
1	8475	Infantile onset	–	3 months	7 months	4 months	–	c.2015G>A, p.R672Q, homozygote
2	8484 8946	Infantile onset	– 18 months	3 months	5 months 2 years	2 months 21 months	–	c.1564C>G, p.P522A, homozygote
3	8668	Infantile onset	17 months	2 weeks	18 months	18 months	–	c.1933G>A, p.D645N, homozygote
4	8557 8868	Juvenile onset	– 6 months	18 years	18 years 19 years	0 1 years	–	c.546+1G>T; n.d.
5	4512 7786	Adult onset	–	40 years	56 years 65 years	16 years 25 years	–	c.-32-13T>G; c.307T>C, p.C103G
6	5638 7774	Adult onset	–	34 years	54 years 60 years	20 years 26 years	50 years	c.-32-13T>G; c.2219delTG, p.V740fsX55
7	5639 7797	Adult onset	–	41 years	42 years 48 years	1 years 7 years	–	c.-32-13T>G; c.546+1G>T
8	6373	Adult onset	–	27 years	28 years	1 years	–	c.-32-13T>G; c.2066_2070duplAGCCG, p.A691fsX6
9	6374	Adult onset	–	32 years	32 years	0	–	c.-32-13T>G; c.2066_2070duplAGCCG, p.A691fsX6
10	7340 9045	Adult onset	– 36 months	50 years	59 years 64 years	9 years 14 years	59 years	c.-32-13T>G; c.2530_2541del, p.A844_L847del

GAA activity in muscle or lymphocytes, and identification of mutations in the *GAA* gene (Nascimbeni et al. 2008). Of the ten patients studied (Table 1), four cases (two Pompe, two adult onset) underwent ERT treatment before the only (patient 3) or a second muscle biopsy was obtained (patients 2, 4, 10), and three untreated adult-onset cases (patients 5, 6, 7) underwent two biopsies during the natural course of the disease.

Muscle Morphology, Immunohistochemistry, and Immunoblot

Muscle biopsy sections were stained using a panel of ten different routine stains (including the lysosomal acid phosphatase), to analyse fibre size and the degree of vacuolisation. We studied muscle autophagy markers and atrophy at different times of the disease progression. For immunohistochemical analysis, muscle sections were incubated with primary antibodies against caveolin-3, p62, LC3, according to methods previously reported (Nascimbeni et al. 2008, 2012). The percentage of vacuolated fibres and p62-positive fibres was defined as those presenting either diffuse or scattered intracytoplasmic vacuoles or staining, respectively. Fibre cross-sectional area (CSA) was measured in at least 200 fibres in each biopsy using ImageJ software.

Results

Patients in Natural History (Table **1**)

Patient 1. This child was born from consanguineous parents and has a first-degree cousin also affected. At 3 months, he presented delayed psychomotor development, high CK (1,260 U/L), axial and limb hypotonia, and poor motility. At age 7 months, he underwent muscle biopsy and molecular diagnosis was obtained. *Patient 8*. This 28-year-old man was the brother of case 9. He had stepping gait and proximal weakness. CK was 3,318 U/L and muscle CT scan showed atrophy in paraspinal and gluteus muscles. Spirometry showed moderate respiratory insufficiency. Muscle GAA activity was 15%. *Patient 9*. This 32-year-old woman had myalgia and elevated CK (825 U/L). Muscle biopsy showed a vacuolar myopathy and GAA activity was 17%. Spirometry revealed slight respiratory insufficiency, and muscle CT showed moderate atrophy of gluteus.

Patients in ERT Treatment (Table **1**)

Patient 2. This female child at 5 months had severe hypotonia, growth retardation, hepatomegaly, and cardiomegaly. Echocardiography showed cardiac hypertrophy and

wall motion abnormalities. CK was 575 U/L. Muscle biopsy showed massive vacuolisation (Fig. 1), and GAA gene mutations were identified. She was ERT treated (20 mg/kg every 3 weeks) only after 8 months. At age 2 years, following *aspiration* pneumonia, she was not able to sit unassisted. A second muscle biopsy at age 2 years showed marked vacuolisation and increased fibrosis (Fig. 2). This patient was a nonresponder to ERT treatment. *Patient 3*. This female child was born at 41 g.w. by caesarean section due to foetal bradycardia. Echocardiography showed bilateral ventricular hypertrophy. CK was elevated. EMG showed myotonic discharges. High levels of urinary Glc4 were found. GAA activity in lymphocytes was reduced. Mild hypotonia and macroglossia were noted. ERT (20 mg/kg every 3 weeks) was started at 20 days of life. Gradual improvement in left ventricular hypertrophy was observed. At 18th month follow-up, a muscle biopsy showed relatively mild myopathic changes with 10% of vacuolated fibres. At 2 years, echocardiography was normal as well as psychomotor development, except for a mild speech delay. *Patient 4*. This 17-year-old boy complained of generalised asthenia and cramps after intense exercise, but was virtually asymptomatic (except for MRC in iliopsoas 4/5). CK was 1,012 U/L. Spirometry showed FVC = 91%, FEV1 = 83%, and pO_2 = 93%. Echocardiography showed mild left ventricular and septal hypertrophy, and ejection fraction was 49.7 %. Muscle biopsy showed glycogen storage with 50% vacuolated fibres (Fig. 1). Muscle GAA was 20%. He had a second biopsy after 6 months of ERT treatment, showing decreased vacuoles and acid phosphatase (Fig. 1). The patient improved after ERT, presenting less fatigue and increased 6-minute walking test. He is a good ERT responder. *Patient 5*. This 40-year-old woman complained of progressive difficulty in climbing stairs, walking, and rising from the floor. At 43 year, she walked with a cane and had marked weakness of iliopsoas and quadriceps muscles and hypotrophy of thighs. ECG was normal. Spirometry showed a slightly restrictive pattern with FVC = 79%. Muscle CT scan showed hypotrophy of gluteus, quadriceps, and posterior thigh muscles. Muscle biopsy at age 56 showed 1% of vacuolated fibres (Fig. 2). Muscle GAA activity was 5%. A second biopsy at age 65 was done, and then, ERT treatment was started. Her 6-minute walking test improved from 80 to 160 m. FVC was 1.8 and 1.6 L after 6 months (Angelini et al. 2009). After 4 years of ERT, she still presented a stable condition. *Patient 6*. This woman had difficulty walking, climbing stairs, and rising from the floor since 34 years of age. Since 50 years, she had progressive respiratory insufficiency and FVC decreased 13% every year. She then used overnight oxygen and had dyspnea at rest. At age 54, a first muscle biopsy showed 20% vacuolated fibres (Fig. 2). Muscle GAA activity was 7%. Muscle CT scan showed marked proximal atrophy in posterior thigh muscles. At age 60, a second biopsy was done. Then, ERT was started. At age 64, she had lordotic gait, was able to climb stairs and to rise from a chair, but could not lift legs. MRC on deltoid was 3/5, on triceps 4/5, external shoulder rotators 3/5, and iliopsoas and quadriceps 3/5. Her 6-minute walking test was 120 m before ERT, 160 m. after 6 months ERT, and returned to 110 m after 12 months. FVC was initially 0.5 L and remained stable after 12 months ERT (Angelini et al. 2009). *Patient 7*. This 43-year-old woman, since 7 years complained of weakness, could rise from the floor only using two hands and climb stairs only using the rail. A first muscle biopsy showed 3% of vacuolated fibres (Fig. 2). Muscle GAA activity was 12%. A second muscle biopsy was done at age 48 before ERT was started. She had an allergic reaction during ERT treatment, consisting of erythematous thoracic rash, and had to discontinue ERT. *Patient 10*. This 59-year-old woman had onset at age 50, with weight loss and muscle weakness. She needed ventilator since 59 years of age, when she underwent the first muscle biopsy that showed 5% of fibres with vacuoles. Muscle GAA activity was virtually absent. At age 64 years, a second muscle biopsy was done after 36 months of ERT treatment, showing decreased vacuoles and acid phosphatase reaction (Fig. 1). ERT resulted in stabilisation of motor functions, and only nocturnal ventilator support was required (Vianello et al. 2013).

Disease Progression and Autophagy Impairment

Infantile and late-onset patients have different levels of autophagic flux and accumulation of p62-positive protein aggregates. Infantile patients show impaired autophagy, whereas late-onset patients display a correlation between autophagic block and muscle atrophy with disease progression (Fig. 2). The comparison of three adult patients who underwent two biopsies during natural history (Table **1**, Fig. 2) showed a different degree of autophagy impairment as revealed by accumulation of p62 protein aggregates. Cross-sectional area (CSA) decreased in all patients in the second biopsy. Patient 7 showed a time-dependent increase of abnormalities: in the first biopsy, she had an almost normal fibre morphology, and in the second biopsy, there was a 60% decrease in CSA, which correlated strikingly with the increase in p62-positive aggregate and vacuolated fibres (from 2 to 40%) (Fig. 2).

Early ERT Treatment Restores Autophagy

One infantile patient (patient 2) and two adult patients (patient 4, 10) were ERT treated for 16, 6, and 36 months, respectively, and after treatment, a second biopsy was taken.

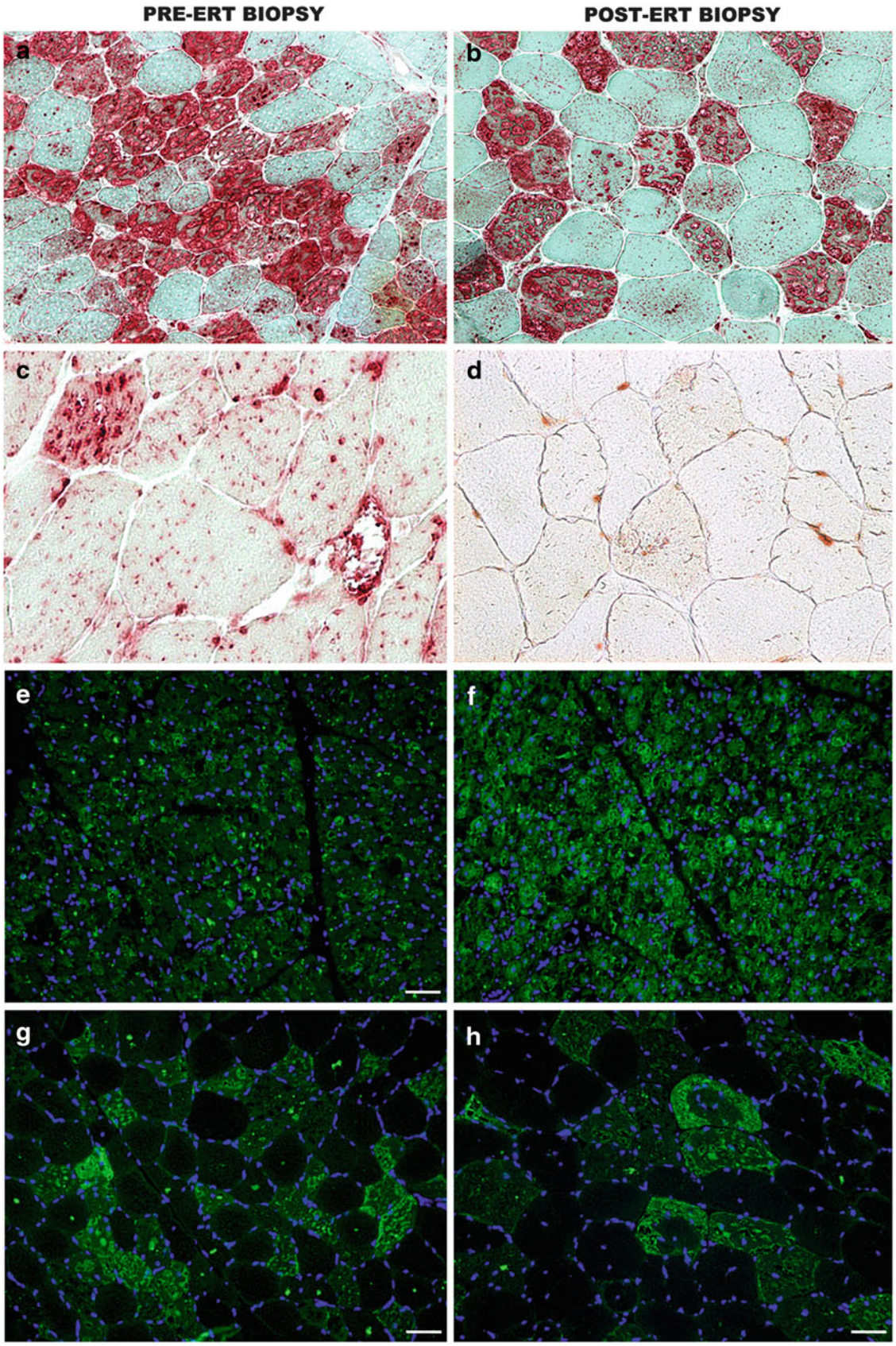

Fig. 1 Comparison of pre-ERT (**a**, **c**, **e**, **g**) and post-ERT muscle biopsies (**b**, **d**, **f**, **h**) stained for acid phosphatase (**a**–**d**) and immunolabelled for p62 protein aggregates (**e**–**h**) in adult early-treated patient 4 (**a**, **b**, **g**, **h**), in late-onset patient 10 (**c**, **d**), and in

The juvenile patient 4 showed a low level of mature GAA that increased after ERT. ERT treatment in this patient greatly reduced the number of p62-positive and vacuolated fibres from 60 to 34% and from 44 to 35%, respectively (Fig. 1), which remained atrophic. MRC of iliopsoas muscle increased from 4 to 5 and his 6-minute walking test also improved. The adult patient 10, after ERT, had less degree of fibre vacuolisation and acid phosphatase reaction (Fig. 1). Consistently, with morphology, therapy in adult patients resulted in less fatigue and better exercise performance.

The infant patient 2 started therapy at 8 months old, an age probably too late for successful recombinant GAA uptake. Indeed, the second biopsy suggested a persistent autophagic impairment despite ERT treatment: p62-positive aggregate fibres increased from 70 to 98%, while vacuolated fibres were 94% before ERT and 98% after ERT (Fig. 1). This patient had no detectable GAA mature forms and instead elevated levels of the inactive 110 kDa precursor (Nascimbeni et al. 2012). ERT induced the appearance of mature 76 kDa GAA even if at very low level. The p62-positive aggregate accumulation and number of vacuolated fibres were increased, possibly contributing to the poor ERT response.

Another early-treated infantile patient (patient 3), who underwent only one biopsy after 18 months of ERT, showed normal CSA value and only few fibres with p62 aggregates (6%) or vacuoles (3%). Also in this case, we can conclude that early treatment, prior to massive autophagy-build-up onset, is effective in restoring the autophagy flux.

Discussion

Autophagy is a highly conserved homeostatic process for lysosome-mediated degradation of cytoplasmic components, including damaged mitochondria and toxic protein aggregates. In infantile Pompe patients, the enormous build-up of glycogen-filled lysosomes appears to cause the muscle damage (Raben et al. 2007). Recent studies showed that autophagy impairment contributes to both disease progression and fibre atrophy (Nascimbeni et al. 2012). A residually functional autophagic flux is important for an efficient ERT delivery in muscle lysosomes, since mature GAA forms were found in our ERT-responsive juvenile case. The maturation steps of GAA, from the synthesis of the immature protein in the endoplasmic reticulum to the final cleaved active protein in lysosomes, are complex and require a functional system of vesicle trafficking.

A limited number of ERT trials and observational studies have been published on late-onset patients (van der Ploeg 2010; Strothotte et al. 2010; Angelini et al. 2012; Regnery et al. 2012). An observational study (Deroma et al. 2014) in eight juvenile GSDII cases reported marked decrease of CK after ERT and stabilisation of disease course. According to treating clinicians, not all patients respond to therapy to the same extent and in the same time frame (Angelini et al. 2012; Strothotte et al. 2010; Regnery et al. 2012). A number of prognostic factors were observed or suspected, including patient gender, age, body composition, genotype, disease duration, and clinical conditions. Good clinical condition and short disease duration seem to be the most important predictors of good response (Angelini et al. 2012; Regnery et al. 2012; Deroma et al. 2014). The majority of infantile patients in whom ERT was started before the age of 6 months and before the need of ventilator support improved and showed longer ventilator-independent survival, reduced cardiac mass, and acquisition of motor skills. Infants who started treatment before the occurrence of extensive muscle damage had better motor outcomes than patients who began treatment at more advanced stages. The same parameters seem to apply to early-treated juvenile cases, where often the main feature is hyperCKemia. At the same time, ventilatory-dependent patients fail to achieve independence from ventilation. Effectiveness of ERT seems to be more limited in advanced cases, even if clinical stabilisation in motor and respiratory function was observed in some advanced cases (Regnery et al. 2012).

The muscle structure is more severely affected in the infantile form, whereas the degree of vacuolisation is variable in late-onset patients. A further difference between infantile and late-onset form is vacuolar compartmentalisation by membranes with sarcolemmal proteins, as shown by caveolin-3 staining (Nascimbeni et al. 2008); this additional feature could be important in determining response to ERT. An important pathological feature is failure of autophagosomal turnover and massive autophagic build-up in fibres. This contributes to myofiber atrophy that increases during natural history in late-onset GSDII (Nascimbeni et al. 2012).

Our data suggest that functional autophagy might protect myofiber from disease progression and muscle atrophy in

Fig. 1 (continued) late-treated infantile patient 2 (**e**, **f**). The early ERT treatment resulted in a reduction of fibre vacuolisation and lysosomal acid phosphatase reaction, suggesting a lower degree of lysosomal impairment. When started early, before autophagy block onset, ERT is efficient and restores the autophagy block, as evidenced by a reduced number of p62-positive fibres in patient 4, but not in patient 2. Bar = 40 μm

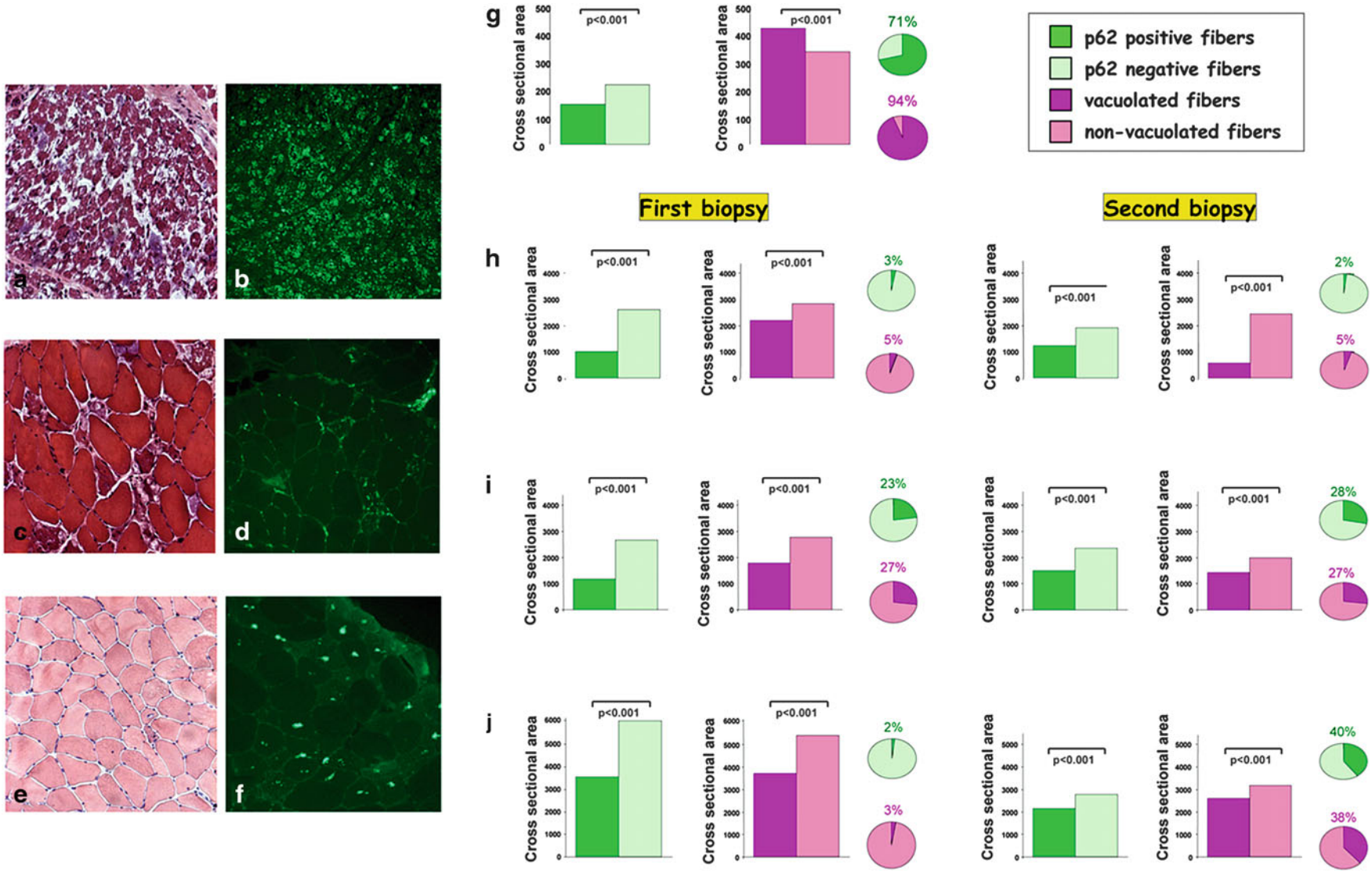

Fig. 2 H&E staining (**a**, **c**, **e**) shows a different degree of muscle fibre vacuolisation in different forms of GSDII. The majority of fibres are vacuolated in the infantile patient 2 (**a**), whereas vacuolated fibres correspond to atrophic fibres in patient 6 (**c**). Muscle biopsy shows mild myopathic changes, consisting of central nuclei and atrophic fibres in patient 5 (**e**). The immunohistochemical analysis of p62-positive aggregates (**b**, **d**, **f**) showed that the majority of fibres present p62 aggregates in infantile patient 2 (**b**) and that most of p62-positive fibres are atrophic and vacuolated in adult patients 6 (**d**) and 5 (**f**). The comparison of fibre cross-sectional areas between p62-positive and negative fibres and between vacuolated and non-vacuolated fibres in the infantile-onset patient 2 (**g**) and adult-onset patients 5 (**h**), 6 (**i**), and 7 (**j**) showed that vacuolated/engulfed fibres display autophagy-related atrophy, suggesting a key role of impaired autophagy and subsequent autophagosomes accumulation in myofibrillar disorganisation and alteration of endocytic trafficking

late-onset patients. The problem of autophagy and fibre atrophy remains to be addressed. Non-pharmacological therapies and drug therapies promoting cellular clearance (or preventing autophagic build-up) might be a future strategy in GSDII management.

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Synopsis

Muscle fibre atrophy and autophagy are linked in the progression of glycogenosis type 2; ERT might reverse such features.

Compliance with Ethics Guidelines

Conflict of Interest

Dr. Corrado Angelini has received compensation from Genzyme European Registry.

Dr. Anna C. Nascimbeni declares that she has no conflict of interest.

Dr. Marina Fanin declares that she has no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (University of Padova, Italy) and with the Helsinki Declaration of 1975 and revised in 2000.

Informed consent was obtained from all patients (or their parents) for being included in the study.

Contribution of Individual Authors

Drs. Angelini, Nascimbeni, and Fanin have contributed pertinent aspects of the planning, conduct, and reporting of the work described in the article.

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RESEARCH REPORT

Is L-Carnitine Supplementation Beneficial in 3-Methylcrotonyl-CoA Carboxylase Deficiency?

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Abstract *Background*: 3-Methylcrotonyl-CoA carboxylase deficiency (3-MCCd) is an autosomal recessive disorder in the catabolism of leucine. In the present study, we investigated the current and prior medical condition of patients with 3-MCCd in the Faroe Islands and their carnitine levels in blood, urine and muscle tissue with and without L-carnitine supplementation to evaluate the current treatment strategy of not recommending L-carnitine supplementation to Faroese 3-MCCd patients.

Methods: Blood and urine samples and muscle biopsies were collected from patients at inclusion and at 3 months. Eight patients received L-carnitine supplementation when recruited; five did not. Included patients who received supplementation were asked to stop L-carnitine, the others were asked to initiate L-carnitine supplementation during the study. Symptoms were determined by review of hospital medical records and questionnaires answered at baseline and after the intervention.

Results: The prevalence of 3-MCCd in the Faroe Islands was 1:2,400, the highest reported worldwide. All patients were homozygous for the *MCCC1* mutation c.1526delG. When not administered L-carnitine, the 3-MCCd patients ($n = 13$) had low plasma and muscle free carnitine levels, 6.9 (SD 1.4) μmol/L and 785 (SD 301) nmol/g wet weight, respectively. L-Carnitine supplementation increased muscle and plasma carnitine levels to a low-normal range, 25.5 (SD 10.9) μmol/L and 1,827 (SD 523) nmol/g wet weight, $p < 0.01$, respectively. Seven of the thirteen 3-MCCd subjects suffered from self-reported fatigue with some alleviation after L-carnitine supplementation.

Conclusion: 3-MCCd is common in the Faroe Islands. Some symptomatic 3-MCCd patients may benefit biochemically and clinically from L-carnitine supplementation, a more general recommendation cannot be given.

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Introduction

3-Methylcrotonyl-CoA carboxylase deficiency (3-MCCd) (OMIM 210200 and 210210) is a defect in the degradation pathway of leucine. 3-MCCd leads to abnormally high levels of 3-methylcrotonylglycine in urine and 3-hydroxyisovalerylcarnitine in the blood. Increased renal excretion of 3-hydroxyisovalerylcarnitine is a natural way of excreting toxic intermediary metabolites that interfere with normal metabolism at the cost of low plasma carnitine levels (Roschinger et al. 1995). Among organic acidurias, 3-MCCd is the most frequently diagnosed disorder at neonatal tandem mass spectrometry (MS/MS) screening (Koeberl et al. 2003; Schulze et al. 2003; Wilcken et al. 2003; Stadler et al. 2006; Lam et al. 2013). The 3-MCC enzyme consists of two subunits, α and β, encoded by the *MCCC1* and *MCCC2* genes, located at 3q25–q27 and 5q12–q13, respectively (Baumgartner et al. 2001; Gallardo

et al. 2001; Holzinger et al. 2001). Mutations in either gene can lead to 3-MCCd, and more than 130 mutations have been reported (Grunert et al. 2012). 3-MCCd in the Faroe Islands is, to the best of our knowledge, caused by homozygosity for a single mutation in the *MCCC1* gene, the c.1526delG deletion.

Patients with 3-MCCd have low blood carnitine levels (Arnold et al. 2008; Grunert et al. 2012). The most severe forms of 3-MCCd have onset in infancy and include lethal cases (Baykal et al. 2005). Some patients suffer episodes of vomiting, lethargy and muscle weakness. These episodes can lead to metabolic decompensation with seizures, coma and death (Gallardo et al. 2001; Baykal et al. 2005). A variety of other symptoms, mostly neurological, have been reported but most patients are asymptomatic. The clinical picture may show extensive intrafamiliar variation (Visser et al. 2000; Baumgartner et al. 2004; Darin et al. 2007; Dirik et al. 2008; Eminoglu et al. 2009; Grunert et al. 2012). However, most children detected through newborn screening remain asymptomatic (Stadler et al. 2006; Lam et al. 2013; Koeberl et al. 2003).

A population-based voluntary screening programme was initiated in 2009 on the Faroe Islands to detect individuals with abnormally low blood carnitine levels caused by primary carnitine deficiency (PCD) from 2009 to 2011; 26,462 participated (Rasmussen et al. 2014). The screening programme also revealed individuals with secondary carnitine deficiency, including 3-MCCd.

The objective of the present study was to investigate the prior and current health status of Faroese 3-MCCd patients and to quantify blood, urine and muscle levels of carnitine and to investigate the effect of L-carnitine supplementation. We know of no prior studies performed to determine the level of carnitine in muscle tissue in patients with 3-MCCd, nor studies that determine the effect of L-carnitine supplementation in this group of patients.

Currently there are no uniform treatment recommendations for 3-MCCd patients – including whether or not to give L-carnitine supplementation (Arnold et al. 2008). L-Carnitine supplementation of patients with 3-MCCd in the Faroe Islands has generally not been recommended, the rationale being that although the patients have low blood carnitine levels, only few seem to suffer symptoms or are at an increased risk of severe medical complications.

Methods

All seventeen registered adult Faroese 3-MCCd patients were invited to participate. A total of thirteen patients were enrolled (see flowchart in Fig. 1). Ten patients were diagnosed with 3-MCCd in the population screening programme. P3 and P8 were diagnosed as parents to heterozygous children detected through newborn screening. P6, a sibling of P3, was diagnosed through family testing of P3's direct relatives.

The design of the study included baseline evaluation, an intervention and an end of study evaluation after 3 months. Blood, urine and muscle tissue were collected at inclusion and at 3 months. Included participants were divided into two groups – depending on whether they received L-carnitine supplementation. Eight patients were on L-carnitine supplementation when included. They were asked to stop L-carnitine intake after the baseline samples had been collected. These patients had received L-carnitine supplementation for at least 2 years, doses ranging from 1.33 to 6 g daily corresponding to 19–87 mg/kg/daily. A second set of samples were obtained after 3 months without L-carnitine supplementation.

Five participants did not receive L-carnitine at baseline evaluation. These L-carnitine-naive 3-MCCd patients received a fixed oral L-carnitine dosage of 1 g three times daily for 3 months, doses ranging from 33 to 46 mg/kg/daily, which was the time we estimated it would take to reach steady-state levels of carnitine in muscle tissue. New samples were collected at 3 months.

Patients 1, 4, 8 and 9 refused to discontinue L-carnitine supplementation, patient 9 due to pregnancy, and patients 1, 4 and 8 were concerned about negative health consequences. Patient number 11 stopped L-carnitine supplementation before we were able to obtain new samples from him due to miscommunication. Patients 2, 3, 5, 6, 7, 10, 12 and 13 are represented in both groups.

Diagnosis was confirmed by DNA analysis performed in the Centre for Inherited Metabolic Diseases (CIMD), Department of Clinical Genetics, Rigshospitalet, Copenhagen (Denmark). Subjects were also genetically analysed for PCD.

All available hospital medical records were systematically reviewed for admissions and outpatient contacts, and the reasons for referral were grouped into the following groups: symptoms from the central nervous system, cardiopulmonary, gastrointestinal, urogenital and endocrine systems as well as gynaecological, obstetrical and orthopaedic complaints.

All patients answered during baseline evaluation a questionnaire. The questionnaire had six main sections: (1) perceived prior and current health status, (2) possible 3-MCCd symptoms (more often ill than others and/or more sick than others when ill, tendency to vomit when ill with a fever, unexplained fainting spells or cardiovascular symptoms), (3) physical capability (feeling reduced physical capability compared to peers, participation in strenuous physical activities, and participation in strenuous organised sport on a national level), (4) dietary habits (craving for meat), (5) L-carnitine supplementation (dosage, effects and adverse effects), and (6) other medications (dosage and

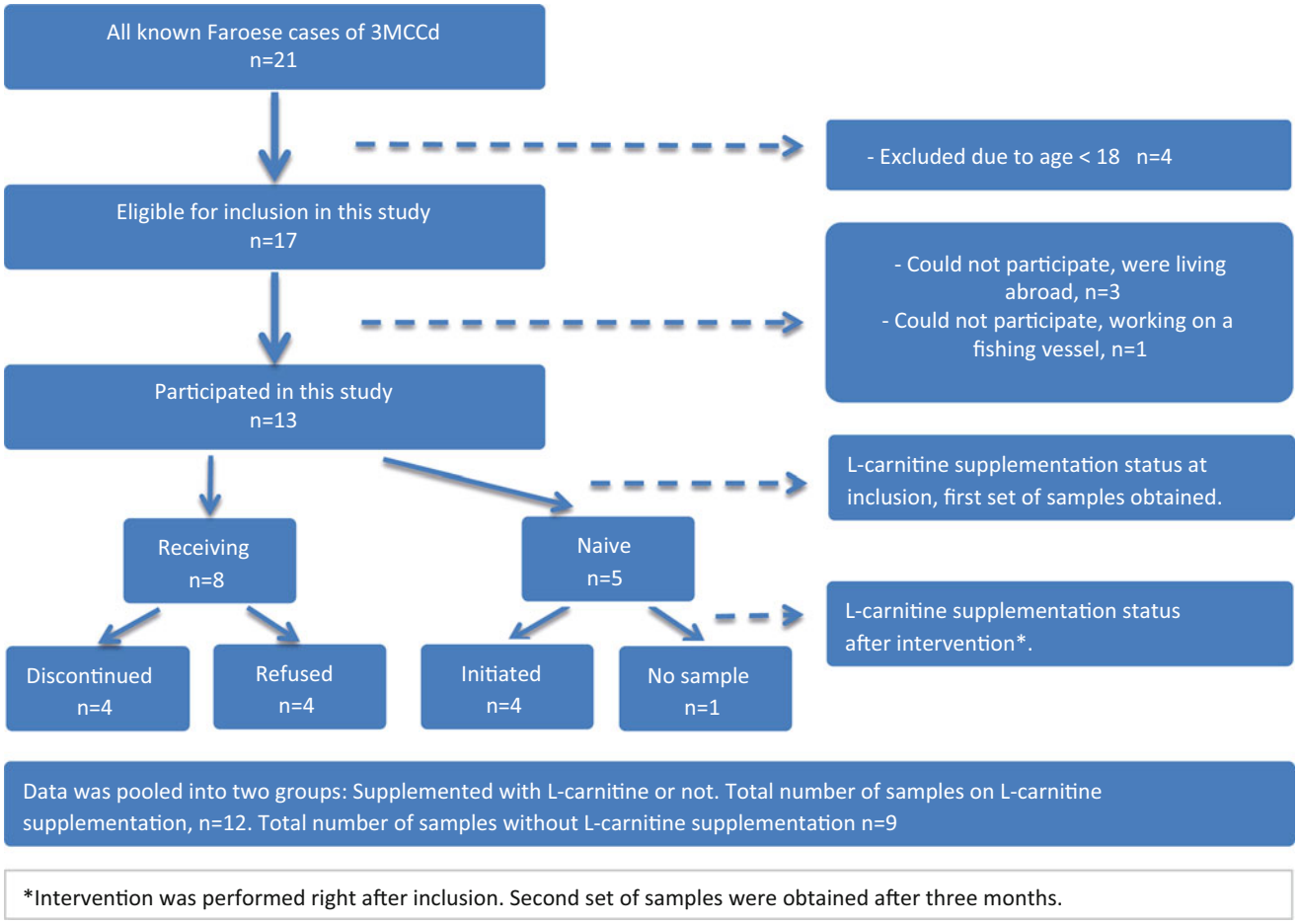

Fig. 1 Inclusion of patients presented in a flowchart

indication stated in free text field). Following the intervention, another questionnaire was answered, which focused on the perceived physical effect of initiation or discontinuation of L-carnitine supplementation. Available answer options were yes, no and unknown except for other medications. Questionnaires were completed by the patients. Symptoms at the time of diagnosis were collected from medical records.

Routine blood samples were analysed in the National Hospital (Faroe Islands) (haemoglobin, mean cell volume, mean cell haemoglobin concentration, leucocytes and leucocyte differential count, thrombocytes, sodium, potassium, creatinine, urea, uric acid, lactate dehydrogenase, creatine kinase, creatine kinase MB isoenzyme, alanine aminotransferase, total cholesterol, high-density lipoprotein, low-density lipoprotein, c-reactive protein), while plasma/urine acylcarnitines and urine 3-hydroxyisovaleric acid were determined at CIMD, Denmark. Electrocardiograms (ECG) were analysed using the Minnesota ECG criteria (Prineas 1982). Echocardiography was performed initially in all patients according to current guidelines (Lang et al. 2005).

Muscle biopsies were taken from the medial part of the m. vastus lateralis using the Bergstrom technique (Bergstrom 1975). The procedure was performed under controlled conditions by a trained biopter. Biopsies were taken with local anaesthesia from the thigh. One elderly male subject on carnitine supplementation had highly atrophic thigh muscles probably due to old age. The subject was biopted thrice, but the samples contained only fat and connective tissue, which is not an uncommon finding in biopsies at old age. The samples were analysed at CIMD, Denmark, to determine the muscle content of carnitine.

Analyses of acylcarnitines and carnitine in plasma, muscle and urine were performed using stable-isotope dilution combined with ultra-performance liquid chromatography–tandem mass spectrometry, using a Quattro Micro triple quadrupole mass spectrometer (Waters, Milford, Massachusetts). d_3-Carnitine, d_3-acetylcarnitine, d_3-propionylcarnitine, d_3-butyrylcarnitine, d_9-isovalerylcarnitine, d_3-octanoylcarnitine, d_3-tetradecanoylcarnitine and d_3-hexadecanoylcarnitine (Hermen ten Brink, Vrije Universiteit, Amsterdam, the Netherlands) were added to samples before extraction/homogenisation. Carnitine and acylcarnitines in all three matrices

Table 1 Blood, plasma, urine and muscle data

Sample site	Sample	Unit	Carnitine supplementation Mean (SD)	No carnitine Mean (SD)	Stud. *t*-test[a] *p*-values
Blood	Haemoglobin	mmol/L	8.1 (0.8)	8.5 (0.6)	0.19
	Potassium	mmol/L	3.8 (0.3)	3.7 (0.2)	0.41
	Creatinine	μmol/L	71.8 (10.9)	78.2 (19)	0.34
	Urea	mmol/L	4.3 (1.1)	4.1 (1.2)	0.73
	ALAT	U/I	22.2 (9.7)	24.2 (17)	0.34
	Random blood glucose	mmol/L	4.9 (0.8)	5.2 (0.5)	0.84
	LDL	mmol/L	3.58 (1.2)	3.5 (1.2)	0.74
Plasma	Free carnitine	μmol/L	25.5 (10.9)	6.9 (1.4)	<0.002
	Acetylcarnitine	μmol/L	5.3 (3.8)	0.8 (0.2)	<0.002
	3-HIV-carnitine	μmol/L	17.3 (3.8)	10.1 (4.8)	<0.002
	Total carnitine	μmol/L	49.6 (15.9)	20.1 (9.4)	<0.002
	Free/total	%	49.6 (8.9)	41.7 (5.4)	0.029
Urine	Creatinine	mmol/L	12.7 (7.4)	14.7 (6.2)	0.52
	Free carnitine	μmol/mmol creatinine	24.2 (32)	2.3 (0.8)	0.056
	Acetylcarnitine	μmol/mmol creatinine	5.3 (7.5)	0.1 (0.03)	0.053
	3-HIV-carnitine	μmol/mmol creatinine	155.5 (68.5)	52.2 (14.7)	<0.002
	3-HIVA	mmol/mmol creatinine	2.2 (0.9)	1.9 (0.5)	0.37
	Total carnitine	μmol/mmol creatinine	184.7 (81)	57.1 (13.7)	<0.002
	Free/total	%	7.8 (8.9)	4.4 (2.2)	0.29
Muscle[b]	Free carnitine	nmol/g wet weight	1,827 (523)	785 (301)	<0.002
	Acetylcarnitine	nmol/g wet weight	195 (55)	86 (26)	<0.002
	3-HIV-carnitine	nmol/g wet weight	2,112 (746)	1,152 (593)	<0.002
	Total carnitine	nmol/g wet weight	4,288 (1,161)	2,117 (800)	<0.002
	Free/total	%	43 (8)	38 (9)	0.18

[a] Student's *t*-test. At $p < 0.05$, difference between groups was significant
ALAT alanine aminotransferase, *LDL* low-density lipoprotein, *3-HIVA* 3-hydroxyisovaleric acid, *3-HIV-carnitine* 3-hydroxyisovalerylcarnitine
Carnitine supplementation group, all patients except P11; [b] no relevant muscle biopsy obtained from P1. No supplementation group: P2, P3, P5, P6, P7, P10, P11, P12 and P13

were quantified using external spiked plasma calibration curves.

Quantitative urine analysis for 3-hydroxyisovaleric acid was performed by stable-isotope dilution combined with GC–MS (HP 6890 GC coupled to a HP5973 mass selective detector) using a d6-3-hydroxyisovaleric acid (Hermen ten Brink, Vrije Universiteit, Amsterdam, the Netherlands).

Statistics and Analysis

Data are presented as mean and standard deviation in parenthesis. Two-tailed Student's *t*-test was used when analysing differences in mean values of routine blood samples and plasma, urine and muscle carnitine values in two groups, with ($n = 9$) and without ($n = 12$) L-carnitine supplementation. Level of significance was $p < 0.05$. The method of Bonferroni was used to correct for repeated testing, the Bonferroni critical value was $0.05/24 = 0.0021$, corrected *p*-value, $p < 0.002$. Correlation between plasma and muscle free carnitine values was calculated with Pearson's test for correlation. Results were computed with Statistical Package for the Social Sciences (SPSS).

Informed consent was obtained from all patients for being included in the study and the study was approved by the Faroese Ethical Committee.

Results

Plasma and muscle free carnitine levels increased significantly when oral L-carnitine was given, $p < 0.01$. Mean plasma free carnitine increased from 6.9 (1.4) to 25.5 (10.9) μmol/L and mean muscle free carnitine increased from 785 (301) to 1,827 (523) nmol/g wet weight after L-carnitine supplementation (Table 1).

There was a significant positive correlation between plasma and muscle free carnitine irrespective of L-carnitine supplementation, $R^2 = 0.657$, $p < 0.01$ (Fig. 2).

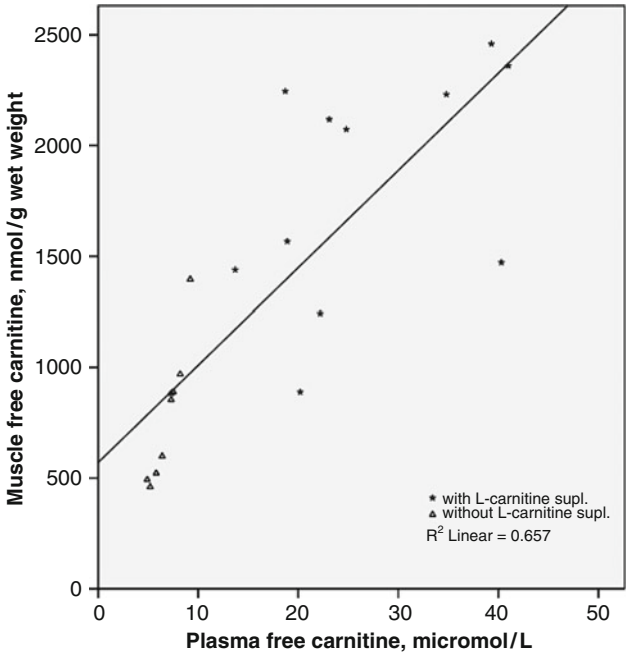

Fig. 2 Plasma free carnitine plotted against muscle free carnitine irrespective of L-carnitine supplementation. There was a significant positive linear correlation, $R^2 = 0.657$, $p < 0.01$

Changes in levels of urine free carnitine and acetylcarnitine demonstrated a tendency towards significance, $p = 0.056$ and $p = 0.053$, respectively, while levels of 3-hydroxyisovalerylcarnitine and total carnitine increased on oral L-carnitine supplementation, $p < 0.01$ (Table 1). Routine blood samples did not differ before and after L-carnitine supplementation (Table 1). Quantitative urine analysis demonstrated no difference between groups regarding levels of 3-hydroxyisovaleric acid, $p = 0.37$ (Table 1).

Review of hospital medical records revealed largely unremarkable medical histories, including 18 live births, three spontaneous abortions and admissions with vertigo, pneumonia, pulmonary embolism, heart palpitations and an extrauterine pregnancy (Table 2). Furthermore, P1 is followed regularly for non-insulin-dependent diabetes mellitus and gout. Baseline characteristics including blood pressure, heart rate, routine blood samples, ECG (electrocardiogram) and transthoracic echocardiography were unremarkable (Tables 1 and 2). Symptoms at the time of diagnosis ranged from none to fatigue and palpitations (Table 2). Mean BMI (body mass index) was 23 and 22 kg/cm^2 and mean age was 49 and 38.3 in male and female 3-MCCd patients, respectively (Table 2).

Six of 13 patients reported chronic fatigue that was alleviated by L-carnitine supplementation. One patient, who did not perceive fatigue at baseline, reported feeling more fit when given L-carnitine. Three patients reported fatigue from childhood. Four patients reported heart palpitations. Two patients stated a tendency to vomit when ill with a fever both in childhood and as adults. Six patients reported participation in strenuous physical activities on a regular basis. Four patients reported a stronger than normal craving for meat. Few side effects of L-carnitine supplementation were reported – one though experienced weight gain and developed an unpleasant body odour (Table 2).

A total of 21 patients, including children and adults, have been diagnosed with 3-MCCd in the Faroe Islands. All were homozygous for a single deletion, c.1526delG, in the *MCCC1* gene. On January 1, 2014, the population of the Faroe Islands was 48,308 (Faroese Board of Public Health 2010). In the screening period from 2009 to 2011, 11 3-MCCd patients were diagnosed from the 26,462 samples collected. Thus, the prevalence of 3-MCCd is 1:2,400. A total of 34,000 live births were recorded in the Faroe Islands from January 1970 until December 2012. During this period, 13 patients with 3-MCCd were born – giving an incidence of 1:2,615 live births.

Subject number 13 was found to be a carrier for the PCD-related c.95A>G (p.N32S) mutation in the *SLC22A5* gene.

The patient cohort includes three sib pairs, P3 and P6, P4 and P8 and P7 and P11.

Discussion

The incidence of 1:2,615 and prevalence of 1:2,400 of 3-MCCd in the Faroe Islands are far greater than reported elsewhere: 1:41,676 in California (Schulze et al. 2003; Wilcken et al. 2003; Lam et al. 2013), 1:64,000 in North Carolina (Koeberl et al. 2003) and 1:84,700 in Bavaria in South Germany

Table 2 Baseline characteristics including ECG and TTE

ID	Age	Gender	Genetic status c.1526delG	fC0[a] µmol/L	BMI kg/m2	Sys./dia. BP mmHg	ECG Changes	TTE		At diagnosis Symp.[b]	Effect of L-carnitine[b]	Medical history Previous admissions
								LVMI (g/m2)	LVEF %			
P1	69	Male	+/+	8	23	157/82	None	83.9	58	Fatigue	Relieved fatigue	None
P2	20	Female	+/+	7	22	123/70	None	68.6	59	Fatigue	Relieved fatigue	None
P3	39	Female	+/+	5.3	24	106/58	None	62.9	59	Fatigue	Relieved fatigue	Ectopic pregnancy
P4	46	Male	+/+	6	26	133/96	None	70.9	55	Palpitations	None	Vertigo, chest pain
P5	46	Female	+/+	4	21	105/62	None	83.5	54	Palpitations	None	Palpitations
P6	49	Female	+/+	5	23	135/83	None	55.1	58	Fatigue	Relieved fatigue	None
P7	43	Female	+/+	8.2	21	116/76	None	63.3	53	Fatigue, often ill	Relieved fatigue	Gallstone
P8	35	Female	+/+	4.3	20	117/69	None	58	62	Fatigue, palpitations	Relieved fatigue, less sick	Spontaneous abortion
P9	22	Female	+/+	4	22	127/69	None	71.7	56	Palpitations	Relieved fatigue	Spontaneous abortion
P10	18	Male	+/+	4	19	141/76	None	82.7	56	Vomiting when sick	None	None
P11	64	Male	+/+	6.4	23	147/85	None	74.2	56	None	None	Pneumonia, PE
P12	52	Female	+/+	5.8	20	121/82	None	69.5	53	None	None	Spontaneous abortion
P13	48	Male	+/+	5.2	25	113/63	None	109.7	58	Vomiting when sick	None	None
Group mean values for numeric values												
Male	49	–	–	5.9	23	138/80	–	84.3	57	–	–	–
Female	38.3	–	–	5.5	21	119/71	–	66.6	57	–	–	–
All	42.4	–	–	5.6	22	126/75	–	73.4	57	–	–	–

[a] Measured pretreatment with L-carnitine when diagnosed during the population screening
[b] Based on medical journal review and questionnaires
fC0 free plasma carnitine, *BMI* body mass index, *Sys.* systolic, *dia.* diastolic, *BP* blood pressure, *ECG* electrocardiogram, *TTE* transthoracic echocardiography, *LVMI* left ventricle mass index (normal value = <115 for males and <95 for females), *LVEF* left ventricular ejection fraction (normal value= > 55), *Symp.* symptoms, *PE* pulmonary embolism

(Stadler et al. 2006). The listed prevalences and incidences are conservative estimates, because we only report on carnitine-deficient 3-MCCd cases and not everyone in the Faroe Islands participated in the voluntary screening programme. The Faroese population stems from a genetic isolate and expanded rapidly during the last three centuries with an almost tenfold increase, from 5,000 to 48,308 individuals, making a founder effect a probable cause of the high prevalence of 3-MCCd in the population (Jorgensen et al. 2002).

We found that all Faroese 3-MCCd patients were carnitine depleted when not treated with L-carnitine, with values of free carnitine in plasma ranging from 4 to 8.2 μmol/L. Free carnitine in plasma increased, $p < 0.01$, when 3-MCCd patients were supplemented with oral L-carnitine, indicating that oral L-carnitine supplementation of 3-MCCd patients can restore levels of free carnitine in plasma to lower range normal values.

We have demonstrated that mean muscle free carnitine increased from 785 to 1,827 nmol/g wet weight of muscle tissue when the patients were supplemented with L-carnitine. Reference mean levels in normal subjects were reported by Opalka et al. (2001) and Madsen et al. (2013) to be 2,400 (800) and 2,914 (249) nmol/g wet weight of muscle tissue, respectively. The difference in reported mean values by Opalka et al. and Madsen et al. might have been caused by an age difference between the cohorts – as mean muscle free carnitine levels reportedly decrease with age (Opalka et al. 2001). The levels of mean muscle carnitine in 3-MCCd patients treated with L-carnitine are in the lower range of the normal reference interval reported by Opalka et al., but below that found by Madsen et al. Mean muscle carnitine reported by Madsen et al. was quantified by the CIMD as in the present study, but on a younger study population (age 19–31), which may explain the lower values in our older cohort compared to the reference interval. We conclude that a significant increase in muscle free carnitine to a low to low-normal level can be obtained by administration of L-carnitine when given for 3 months or more.

We have demonstrated that rising plasma values of free carnitine are an indicator of rising intramuscular free carnitine levels in 3-MCCd patients (Fig. 2) as there was a significant positive correlation between increasing plasma carnitine levels and an increase in the level of carnitine in muscle – the principal store of carnitine in the body. Patients with a secondary carnitine deficiency such as 3-MCCd have a normally functioning carnitine transporter and are thus able to transport more carnitine into the intramuscular compartment when plasma carnitine levels increase. We conclude that plasma levels of free carnitine reflect the intramuscular levels and can be used as a marker for intramuscular levels of free carnitine.

Review of hospital medical records and questionnaires revealed unremarkable present and past medical histories concerning serious illnesses or diseases. Furthermore, routine blood samples did not differ before and after L-carnitine supplementation. Baseline ECGs, blood pressures and transthoracic echocardiograms were unremarkable as well. The reported clinical picture in the literature is heterogeneous, ranging from fatal cases (Gallardo et al. 2001; Baykal et al. 2005) to asymptomatic adult cases (Gallardo et al. 2001) including mothers diagnosed due to abnormal newborn screening results in their infants (Grunert et al. 2012). Clinical presentation varies even within families (Visser et al. 2000; Eminoglu et al. 2009). Neurological abnormalities have been reported (Baykal et al. 2005) including one case of multiple sclerosis (Darin et al. 2007). The mother of siblings P4 and P8 suffered multiple sclerosis – however, the patients and their brother did not present or complain of neurological deficits.

Carnitine depletion in PCD has been linked to sudden death (Rasmussen et al. 2012). In PCD, the organic cation transporter 2 (OCTN2) is deficient and leads to intracellular carnitine depletion in contrast to the situation in patients with 3-MCCd who have a normal OCTN2 activity that will probably secure a sufficient intracellular carnitine level and prevent sudden death.

Though their medical histories were unremarkable, some patients reported mild but long-lasting symptoms. Fatigue was the main symptom reported by 54% of the patients. Given the small sample size and the difficulties in grading fatigue even in large populations, the research group chose not to include a formal grading of the self-reported symptoms of fatigue. Grunert et al. (Grunert et al. 2012) reported that among 88 patients, only one patient (#87) or 1.1% reported suffering from chronic tiredness – patient #87 was Faroese and also included in our study as P8. The prevalence of fatigue was thus much higher in our cohort. Reasons for the difference might include different interview/reporting procedures between the studies, cultural differences in feeling fatigued and reporting it as a diagnosis and a possible association between the Faroese *MCCC1* mutation c.1526delG and fatigue.

Two patients described consistent vomiting when ill with a fever, which could indicate a tendency to slight metabolic decompensation. Metabolic decompensation with vomiting during illness is well described in cases of organic aciduria (Pasquali et al. 2006).

3-MCCd patients seem slender; mean BMI was 23 and 22 kg/cm^2 for males and females, respectively. A Faroese population health survey from 2010 showed that mean BMI for men and women aged 40 to 70 was 28 and 26, respectively (Statistics Faroe Islands 2014). Thus, the 3-MCCd patients seem to have a normal BMI, though lower than the national average. Our sample size is small and only 8 of 13 patients fall into the sampled age group (Table 2). Lower than national average BMI has not been reported for 3-MCCd by other research groups, but the reason for the finding is unclear and might be due to chance.

The enzymatic activity of 3-methylcrotonyl-CoA carboxylase was not quantified in the present study. It has been reported previously by Grunert et al. (2012) for patient P3, labelled patient #73c, and patient P8, labelled patient #87, as being severely reduced. All cases are homozygous for the *MCCC1* mutation c.1526delG and therefore we expect reduced enzymatic activity in all subjects.

The patients in this study are enzymatically and molecularly homogenous concerning 3-MCCd, which may be a strength when evaluating the clinical consequences of the disease. However, though generally mild, the phenotype varies between the patients, which has also been demonstrated previously within families (Eminoglu et al. 2009), making general conclusions and treatment recommendations less viable and might instead call for future individualised treatment strategies.

Grunert et al. (2012) described 88 3-MCCd patients with varying mutations in either *MCCC1* or *MCCC2*. They found no indication for dietetic treatment (except for maybe an emergency regimen during intercurrent illness) and recommended supplementation with L-carnitine if free carnitine levels are low or patients are symptomatic. Arnold et al. (2008) recommend L-carnitine supplementation for carnitine-deficient cases regardless of symptomatology. Faroese 3-MCCd patients were all carnitine depleted at diagnosis and some had minor symptoms. Based on the treatment strategies proposed by Grunert et al. and Arnold et al. and our finding that some patients experience alleviation of fatigue when treated with L-carnitine, it may be discussed whether L-carnitine supplementation should be recommended to Faroese 3-MCCd patients. However, the symptoms experienced by these patients are not severe – rather these patients have been feeling well for extended periods. Data from other patient groups with secondary carnitine depletion (as opposed to those with PCD) are not conclusive concerning a beneficial effect of L-carnitine supplementation, e.g. as in MCADD (medium-chain acyl-CoA dehydrogenase deficiency) patients (Madsen et al. 2013). Supplementation in patients with LCHADD (long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency) on the other hand may cause increased production of toxic 3-hydroxyacylcarnitines (Spiekerkoetter et al. 2009). Possible consequences of long-term L-carnitine supplementation are not to date sufficiently documented, and some data may raise concerns – Koeth et al. suggest a linkage between ingestion of L-carnitine supplementation and cardiovascular disease (Koeth et al. 2013). Furthermore, levels of 3-hydroxyisovaleric acid in urine did not differ between those on and those without L-carnitine supplementation, $p = 0.37$, arguing against an effect of detoxification of the L-carnitine given. It may be reasonable to supplement symptomatic patients, but it seems premature at present to give any more general recommendations.

Limitations

L-Carnitine was administered in a fixed dosage of 1 g three times daily, when provided by the research group. One could argue that doses based on body weight would render a more true reflection of the effect of L-carnitine on intramuscular carnitine levels. Fatigue, the dominating symptom reported in questionnaires, is difficult to score and represents a subjective feeling experienced by patients – however self-reported fatigue that alters quality of life must be taken into consideration and treated as a genuine symptom. In the examined patient category, the patients have had a lifelong state of secondary carnitine depletion. Quantification of fatigue in the presence of this baseline state is inherently difficult, as demonstrated in this study by the fact that a patient upon L-carnitine supplementation reported alleviation of fatigue, which the person had not been conscious of and had not reported previously.

Conclusion

3-MCCd due to the *MCCC1* c.1526delG mutation is common in the Faroe Islands compared to the rest of the world because of a probable founder effect. Levels of free carnitine in muscle tissue and blood were low in patients without L-carnitine supplementation and increased significantly to low–low-normal levels upon L-carnitine supplementation. Plasma levels of free carnitine and intramuscular levels are highly correlated regardless of carnitine level; therefore plasma carnitine levels can be used as an indicator for intramuscular levels. Seven of 13 investigated patients presented with a subjective feeling of fatigue that was alleviated after L-carnitine supplementation. Taking into consideration the newly raised concern regarding the safety of L-carnitine supplementation, the absence of dangerous symptoms and the significant but mild subjective feeling of fatigue reported by some patients, a general recommendation about supplementing all 3-MCCd patients with L-carnitine cannot be given. However, since L-carnitine supplementation can alleviate fatigue suffered by some patients, one could argue for an L-carnitine supplementation trial for these selected patients.

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Synopsis

Intramuscular levels of free carnitine correlate with plasma levels of free carnitine and can be restored to near-normal levels with oral L-carnitine supplementation in 3-MCCd patients.

Compliance with Ethics Guidelines

Conflict of Interest

Jákup Andreas Thomsen, Allan Meldgaard Lund, Jess Have Olesen, Magni Mohr and Jan Rasmussen declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

Details of the Contributions of Individual Authors

Corresponding author Jákup Andreas Thomsen was involved in all aspects of the work and is the guarantor and wrote the article.

Jan Rasmussen has been involved in the conception, design, analysis and interpretation of data and revised the article critically for important intellectual content.

Magni Mohr has been involved in the conception, design and interpretation of data and revised the article critically for important intellectual content.

Jess Have Olesen has been involved in the conception, design, analysis and interpretation of data and revised the article critically for important intellectual content.

Allan Meldgaard Lund has been involved in the conception, design, analysis and interpretation of data and revised the article critically for important intellectual content.

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RESEARCH REPORT

Neurodevelopmental and Cognitive Outcomes of Classical Homocystinuria: Experience from Qatar

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Abstract *Background*: Classical homocystinuria due to cystathionine β-synthase (CBS) deficiency (OMIM 236200) is a recessively inherited condition caused by mutations in the *CBS* gene. The founder mutation p.R336C accounts for almost all CBS deficiency in Qatar, affecting approximately 1 in 1,800 births, making it the most prevalent monogenic disease among the Qatari population. Untreated patients can have severe intellectual disability (ID), devastating multisystem complications and premature death. Current treatment is based on pharmacology therapy and life-long methionine-restricted diet, which is difficult to maintain particularly in late diagnosed individuals. Data on the neurodevelopmental and psychological impact of the disease on outcomes among Qatari patients are generally lacking and have not been studied.

Objectives: To examine the cognitive, educational and psychological outcomes of classical homocystinuria on Qatari patients.

Subjects and Methods: Thirty-two cases with classical homocystinuria and 25 sibling controls were recruited to evaluate the neurodevelopmental and cognitive outcomes. We reviewed the subjects' medical record and collected pertinent clinical and educational data from parents. Stanford–Binet Intelligence Test (Arabic translation – 4th ed.) was used for cognitive (IQ) testing.

Results: The mean age for the subjects was 11.2 years (range 0.6–29) with 56% males. The majority of cases (93%) carried the mutation (p.R336C), and parental consanguinity was 84%. There were no differences between the two groups in the fine motor, expressive language, behavioural and visual skills. However, cases have much lower total IQ particularly in the domains of short memory, quantitative reasoning and visual–spatial domains. A significant number of adolescents and adult cases had medical co-morbidities as well as behavioural and emotional problems.

Conclusion: Individuals with classical homocystinuria have many developmental and cognitive difficulties with significant number of cases having learning disability and lower IQs (cf. sibling controls) with adolescents and adults more affected. Those diagnosed by newborn screening have better developmental and cognitive outcomes compared to late diagnosed cases. Psychological and psychiatric referrals should be part of the standard of care for those cases

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Introduction

Classical homocystinuria (CHU) due to CBS deficiency (OMIM 236200) is caused by mutations in the *CBS* gene. Although this disease is the most frequent disorder of sulphur and methionine metabolism, it is still considered a rare inborn error of metabolism with estimated prevalence between 1/20,000 and 344,000. However, in Qatar, its prevalence is extremely high, the highest in the world, of approximately 1/1,800 births (Mudd et al. 1964; Mudd 1985; Naughten et al. 1998; El-Said et al. 2006; Zschocke et al. 2009; Gan-Schreier et al. 2010; Yap 2012). Clinically,

Table 1 Mean homocysteine and methionine pre- and posttreatment levels among CHU cases diagnosed by NBS and those of late diagnosis

	Diagnosed by NBS	Late diagnosis
Mean homocysteine level (umol/L)		
Pretreatment	141.6	130.5
Posttreatment	40.8	115.2
Mean methionine level (umol/L)		
Pretreatment	468.9	770.6
Posttreatment	256.1	928.2

CBS-deficient patients may present with ectopia lentis, osteoporosis, and skeletal deformities often associated with Marfanoid features, but most importantly with intellectual disability and life-threatening complications of the vascular system leading to premature death (Mudd 1985; Mudd et al. 1985; Yap 2012). Therapy of homocystinuria usually includes administration of high doses of pyridoxine, the cofactor of CBS; however, only less than 50% of affected subjects show a substantial plasma homocysteine reduction (Mudd 1985; Lindner et al. 2007; Yap 2012). The homocystinuria patients in Qatar are known to be pyridoxine nonresponsive with more severe phenotype and complications (El-Said et al. 2006; Zschocke et al. 2009; Gan-Schreier et al. 2010).

Human CBS is a pyridoxal 5′-phosphate (PLP)-containing enzyme (E.C. 4.2.1.22), and it is the enzyme involved in the first step of the methionine transsulphuration pathway, where Hcy is combined with serine forming cystathionine, which in turn is the precursor of cysteine. Consequently, an impaired CBS activity leads to hyperhomocysteinaemia and homocystinuria, hypermethioninaemia and hypocysteinaemia (Mudd 1985; Yap 2012). The human *CBS* gene, mapped to chromosome 21q21-3, encompasses 30 kb of genomic DNA, and a total of 23 exons have been reported (Lindner et al. 2007). However, only exons 1–14 and 16 encode the CBS protein. More than 140 different disease-causing mutations have been identified in the *CBS* gene, and although scattered along the entire gene length, mutations in exons 3, 8 and 10 are most prevalent (Schiff and Blom 2012). Most of these mutations are missense, and only three nonsense mutations have been reported. In addition, several splicing mutations as well as deletions and insertion mutations have been found (Kraus et al. 1999; Schiff and Blom 2012).

The founder mutation p.R336C (c.1006C>T) can cause a severe B6 nonresponsive phenotype. If untreated, homozygous patients for this mutation (the usual situation in Qatar) are clinically affected with severe intellectual disability as the most prominent feature, in addition to devastating multisystem complications and premature death (El-Said et al. 2006; Zschocke et al. 2009; Gan-Schreier et al. 2010). Furthermore, p.R336C mutation exhibited an activity lower than 4% of the wild-type protein (Urreizti et al. 2006). This disease imposes a huge clinical, financial and psychosocial overall burden on the population of Qatar (Gan-Schreier et al. 2010).

Treatment of patients with CHU whether diagnosed early by newborn screening or later is based on a combined lifelong methionine-restricted diet and pharmacological therapy. Dietary treatment includes low methionine diet with cysteine enriched methionine free amino acid supplement. The use of dietary control and medications is an effective intervention to reduce the mean homocysteine and methionine levels. However, dietary control is rather difficult to maintain, in particular for late diagnosed patients and during puberty, adolescence and adulthood. Pharmacological therapy includes betaine, in addition to vitamin B6, vitamin B12 and folate supplementation. In our cohort of cases with CHU, the overall disease control (measured by homocysteine and methionine levels) is significantly better among those diagnosed through newborn screening than those diagnosed clinically. This is attributed mainly to poor compliance with both diet and medications (Table 1).

In this study, we prospectively assessed the neurodevelopmental and cognitive outcomes in 32 children and adults with CHU and compared them with 25 sibling controls using standardized assessments.

Methodology

Between April 2011 and March 2013, a total of 32 cases with CHU who attended the Metabolic Clinic at Hamad Medical Corporation (HMC), Doha, Qatar, were invited to participate in the study. We also recruited one (the immediate older or younger) unaffected sibling of the same sex to serve as control for the subject. Sibling controls were used as the neurodevelopmental and cognitive outcomes assessed in this study may be affected by family circumstances and genetic makeup.

The diagnosis was achieved in late diagnosis/symptomatic patients by measuring plasma total homocysteine.

The diagnosis is further supported by simultaneously high or borderline high plasma methionine. Confirmation of the diagnosis is by mutation analysis of the CBS gene (common Qatari mutations). For newborn babies (identified by NBS), we measure total homocysteine in dried blood spots by LC/MS/MS. Then, in positive cases, confirmation of the diagnosis is achieved by measuring plasma total homocysteine, plasma methionine and mutation analysis of the CBS gene.

A detailed medical assessment including history, physical examination and medical records review was conducted by the study investigators to collect pertinent data on demographic factors, medical, laboratory, neurodevelopmental, psychosocial and educational attainment. Direct questioning of parents and or adult cases was used to collect pertinent data that was not available in the medical records.

Cognitive assessment was conducted using Stanford–Binet Intelligence Scale – 4th ed. (Arabic translation) by a trained clinical psychologist. The test yields a total mental processing composite based on subtests that measure verbal reasoning, quantitative reasoning, visual–spatial processing, working memory and total IQ. For children under 3 years of age at testing, cognitive assessment was not done.

T-test or Fisher's exact test was used for normally distributed data, and for non-normally distributed data, non-parametric tests were used. Matched and unmatched analysis for all variables was used to adjust for the use of sibling controls.

Differences in baseline characteristics were assessed using McNemar's test for comparing dichotomous variables and chi-square and Fisher's test for higher-order categorical variables. Two-tailed significance test was used with a p-value of <0.05 as significance level. Statistical analyses were conducted in STATA 13.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

Results

Thirty-two cases and 25 sibling controls were available for evaluation. The mean age for the study's subjects (cases and controls) was 11.2 years (range 0.6–29) and 32 (56%) were males. The mean age at diagnosis of all cases was 66 months (range 1–240), and 9 (28%) were diagnosed in the first month of life through the national expanded newborn screening programme that was established in early 2006. The mean age of the 14 (44%) cases that were diagnosed prior to 2006 was 92 months (range 14–240) compared with a mean age of 100 months (range 56–142) for those diagnosed on or after 2006 excluding cases diagnosed by newborn screening (p = NS).

Parental consanguinity was positive in 27 (84%) of the cases. Interestingly, all cases carry the founder mutation p.R336C, except one child who had the G347S mutation.

Developmental Domains

In 25 (78%) of cases, the parents reported one or more concerns about their children's development. Table 2 shows developmental difficulties among cases and controls. Parents reported more difficulties in the domains of fine motor skills and coordination, and expressive language, but only the vision and behavioural/emotional problems were statistically significant when compared with their sibling controls. All cases were able to walk independently; however, three cases needed help to climb stairs or to run. None of them had cerebrovascular insult; however, one child had white matter changes in his brain MRI. There were no significant differences in developmental domains among boys and girls in the study.

There were no parental concerns regarding daily living activities (eating, personal care skills and independent use of toilet), but four cases aged 10, 13, 22 and 29 years have major coordination and handwriting difficulty skills.

Seven cases had both receptive and expressive language difficulties, and in four cases expressive language difficulties were only reported. At the time of enrolment, two children were getting private speech therapy sessions.

A significant number (13/32) of cases were reported by their parents to have behavioural and emotional difficulties. The mean age for those cases was 14.6 years (range 9–29) and six were females. Eight (61%) of the cases had a mean total IQ < 70. According to the families, none of their children had a formal psychiatric assessment or an explicit diagnosis.

Over half of the cases reported to regularly attending the ophthalmology clinics because of their visual difficulties and two needed eye operation. Four cases were labelled as visually impaired and attend a special school for children with visual impairment.

Education Attainment

Table 3 shows the educational attainment history as reported by parents. At the time of enrolment, a significant number of cases were attending or previously attended special needs school or needing extra help in the classroom to access the mainstream education curriculum. A number of parents reported that their child's school performance was average or below what is expected especially in arithmetic and language. Overall, cases and controls had good school attendance record.

Table 2 Developmental difficulties among cases and controls as reported by parents

Domain	Cases ($n = 32$)	Controls ($n = 25$)	p-value
Gross motor skills	3 (9%)	0 (0%)	NS
Fine motor skills + coordination	4 (12.5%)	0 (0%)	NS
Receptive language	7 (22%)	3 (12%)	NS
Expressive language	11 (34%)	3 (12%)	NS
Self-care skills	2 (6.2%)	0 (0%)	NS
Behavioural/emotional	13 (40.6%)	1 (4%)	0.002
Vision problems	18 (56%)	3 (12%)	< 0.001
Hearing problems	0 (0%)	1 (4%)	NS

NS not significant

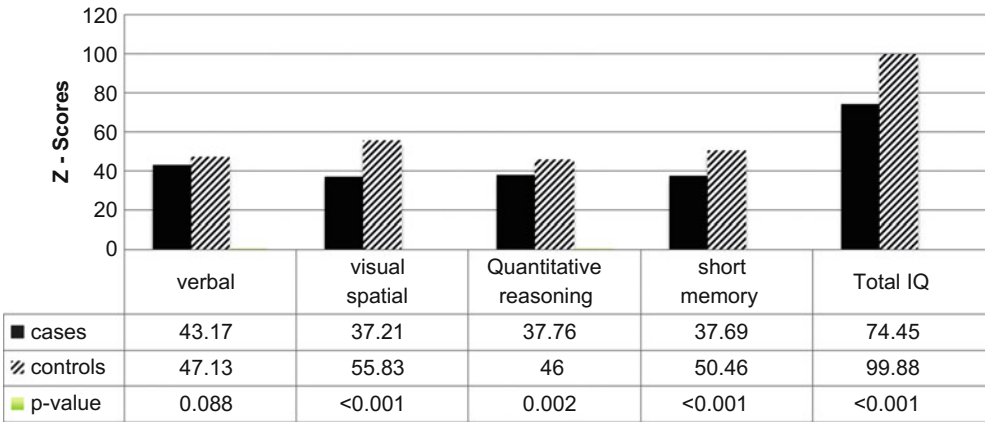

Fig. 1 Total and subscale IQ scores among cases and controls

Cognitive Function

The cognitive data was obtained from 28 cases and 24 controls. Cognitive testing was not available in the remainder of cases and controls due to young age and refusal to conduct the test. Total IQ score as well as three of the four subset scales (quantitative reasoning, visual–spatial processing, working memory) for the Stanford–Binet Intelligence Scale – 4th ed (Arabic translation) were significantly lower among cases compared with controls ($P < 0.001$) (Fig. 1).

Figure 2 shows the classification of cognitive levels according to the total IQ score. Unlike the controls whom the majority (19/24) were classified to have average or high average cognitive level, only 9 (32%) cases had total IQ in the average or high average ($p < 0.001$). Moreover, more than a third of the cases (10/28) had total IQ < 70 and were classified as mental retardation but none of the controls ($p = 0.001$). There were no significant differences in IQ among boys and girls in the study.

Newborn Screening and Outcomes

Table 4 shows the developmental and cognitive outcomes among cases stratified by age at diagnosis. Nine cases were diagnosed within the first month of life by newborn screening, whereas the remainder of the cases were diagnosed at different ages according to their presentation. None of the nine cases had visual problems compared with 18 (78%) in the late diagnosed group. This is a significant difference between the two groups. One child in the newborn screening group has receptive and expressive language difficulties at the time of enrolment; however, his total IQ was 116 (high average).

The differences in language domains, attendance at special school and access to extra support in class were not statistically significant between those diagnosed soon after birth and cases detected/diagnosed later in life. Nonetheless, it is apparent that the "late detection" group had more reported difficulties. In addition, the late diagnosed group

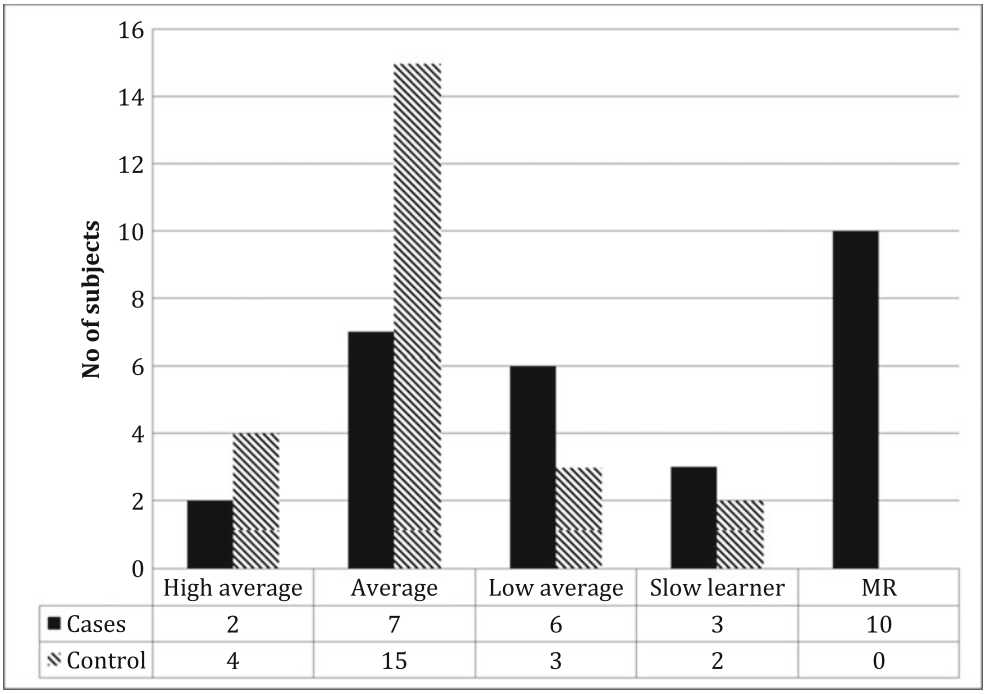

Fig. 2 Classification of IQ scores among cases and controls

Table 3 Education variables among cases and controls as reported by parents

Variable	Cases ($n = 27$)	Controls ($n = 21$)	p-value
Attends education	26 (96.3%)	20 (95.2%)	NS
Attends special needs school or centre	6 (22%)	0 (0%)	0.028
School performance rated by parents as average or below	16 (59%)	9 (43%)	NS
Extra help in class	10 (37%)	3 (14.3%)	NS
$>$ 3 days off school in 3 months	4 (15%)	2 (9.5%)	NS

NS not significant

total mean IQ was significantly lower when compared with the newborn screening group ($p < 0.001$) (Table 4).

Discussion

We conducted a case–control study to report on the neurodevelopmental, educational and cognitive outcomes of cases with CHU. To our knowledge, only a limited number of studies have reported on these outcomes in subjects with CHU. We used siblings as control to adjust for genetic, environmental (protective or risk) factors and socioeconomic status that may affect the outcomes in question especially the IQ which could be highly heritable (Bailey and Revelle 1991; Devlin et al. 1997; Dickens and Flynn 2001; Turkheimer et al. 2003). The use of sibling controls in this study is not novel, as a number of previous studies have used siblings as controls possibly for the same reasons mentioned above (Koch et al. 1984; Eldridge et al. 1989; Yap et al. 2001).

In 2003, Qatar has established an expanded newborn screening (NBS) programme for a number of metabolic and endocrine conditions including CHU (by using methionine) (Lindner et al. 2007; Zschocke et al. 2009; Gan-Schreier et al. 2010). In 2006, NBS for CHU performed by measuring total homocysteine. The introduction of the screening programme has clearly helped in the early detection of newborn cases. All cases bar one were homozygous for the founder p.R336C mutation, and considering the high consanguinity rate among the families, this explains the high prevalence rate of CHU in Qatar (El-Said et al. 2006; Zschocke et al. 2009; Gan-Schreier

Table 4 Age at diagnosis and developmental, educational and cognitive outcomes among the cases as reported by parents

Variable	Newborn screening ($n = 9$)	Late detection ($n = 23$)	p-value
Mean (SD) age at enrolment	4.2 (1.7)	14.0 (6.2)	<0.001
Vision problems	0	18	<0.001
Receptive language difficulties	1	6	NS
Expressive language difficulties	1	10	NS
Special school	0 ($n = 4$)	6	NS
Extra help in class	1	9	NS
Mean (SD) total IQ	101 (14.2)	66 (19.8)	0.002

NS not significant, *SD* standard deviation

et al. 2010). It is interesting that cases reported from a neighbouring country (Saudi Arabia) had different common mutations for CHU (Al-Essa et al. 1998).

Our study showed a large number of families had raised concerns about one or more domains of their child's development, and clearly there was a trend of difficulties among the cases; nonetheless, the data did not show statistical differences between cases and controls in the gross or fine motor skills or the language domains. This could be attributed to the small sample size.

Forty percent of our cases were reported by their parents to have behavioural and emotional difficulties. Our study concurs with previous studies that reported similar or even higher rates of psychiatric/behavioural disorders among cases of homocystinuria (Abbott et al. 1987). Another significant finding is that all those cases bar one had total IQs that are low average or below 70. The association between psychiatric/behavioural problems and low IQ in CHU has been reported previously (Abbott et al. 1987). However, it is not clear from our study if those cases have behavioural/emotional issues secondary to their low cognitive abilities or they have primary behavioural/emotional problems that are independent of their IQ; particularly the parents have denied any formal diagnosis or follow-up of psychiatric illness among the cases. This may be explained by cultural factors or more family support particularly among female patients. A recent systematic review has reported difficulty in assigning causality association of metabolic disorders with psychiatric illnesses in adults (Bonnot et al. 2014).

Visual problems were very prevalent among our cases and clearly represent a long-term morbidity. However, it was reassuring that the majority of cases are regularly followed up and closely monitored in the Ophthalmology Clinic. A significant number of cases have low cognitive abilities as highlighted by their low total and subset IQs. The high prevalence of visual problems may have contributed further to their lower score. Low IQs in the subset of short-term memory and reasoning negatively impact on the child's learning ability.

Our study is susceptible to biases common to case–control studies. It was not possible to blind the study team to the subject's status. Another limitation is the relatively small sample size which could be the reason why many of the differences between cases and controls did not achieve statistical significance; however, CHU is still a rare condition and most of the studies that addressed the neurodevelopmental and cognitive outcomes have published similar number of subjects or even less (Yap et al. 2001; Weisfeld-Adams et al. 2013). Another important limitation is that we relied on parent/subject report and recall on some of the developmental and educational domains. It is not clear if those reports have over- or underestimated the prevalence of difficulties in those domains. Further studies with larger sample size and using assessment tools that have been translated and validated into Arabic language may help to control this limitation.

Conclusion

It is clear that current treatment for CHU in early-treated children, particularly in those detected by NBS, has eliminated severe intellectual and cognitive impairment. More support is needed for cases attending mainstream schools especially those with low cognitive abilities and those with visual problems as they may need extra support in class or individual educational plans that take into account their overall difficulties. Future research assessing intellectual and neurocognitive outcome in CHU will enhance the development of new treatment strategies. Long-term follow-up studies are needed to explore the behavioural/emotional difficulties especially among adolescents and adults with this condition. Psychological and/or

psychiatric referrals should be part of continuing care of those cases especially for those with low cognitive (IQ) abilities who may be at greater risk.

Compliance with Ethics Guidelines

Haitham El Bashir, Lubna Dekair, Yasmeen Mahmoud and Tawfeg Ben-Omran declare that they have no conflict of interest. The study was approved by the Hamad Medical Corporation, Medical Research Center Ethics Committee (# 10263/10).

- Haitham El Bashir: conceived the idea, wrote the study protocol and ethical approval, study design, data analysis, wrote the first draft of manuscript, study guarantor
- Lubna Dekair: study design, recruitment, data collection, developmental assessment, critical review of manuscript
- Yasmeen Mahmoud: study design, recruitment, data collection, cognitive assessment, critical review of manuscript
- Tawfeg Ben-Omran: study design, recruitment of cases, data collection, critical review of manuscript

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RESEARCH REPORT

Diet History Is a Reliable Predictor of Suboptimal Docosahexaenoic Acid Levels in Adult Patients with Phenylketonuria

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Abstract *Background*: Omega-3 long-chain polyunsaturated fatty acids (n3LCPUFA) levels are reduced in phenylketonuria (PKU). Recent care guidelines recommend essential fatty acid status is monitored in patients with PKU but access to such testing is limited. We hypothesized that information obtained on diet history would identify PKU adults with suboptimal levels of n3LCPUFA.

Methods: A 12-month single site prospective study was completed including 35 adults (age 18–46) attending a clinic for adults with inborn errors of metabolism. Levels of n3LCPUFA were correlated with estimated intake using a published food frequency questionnaire. n3LCPUFA levels were tested at a commercial laboratory and values > one SD below the laboratory mean value were considered suboptimal.

Results: Mean levels of docosahexaenoic acid (DHA) were lower and levels of eicosapentaenoic acid (EPA) and alpha-linoleic acid (ALA) higher in subjects with PKU than in laboratory controls. n3LCPUFA levels correlated with estimated intake (p <0.002). Diet history had a positive predictive value of 93% and negative predictive value of 90% to identify subjects with suboptimal n3LCPUFA levels.

Conclusions: Diet history is sufficient to predict adult subjects who may have low DHA levels and can be used to target testing or supplementation to those at risk. DHA levels are low despite high levels of ALA suggesting that supplementation, if indicated, should be with preformed DHA rather than with its precursors.

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Introduction

Suboptimal levels of omega-3 long-chain polyunsaturated fatty acids (n3LCPUFA) have been documented in patients with phenylketonuria (PKU) (Lohner et al. 2013) related both to dietary restriction of sources of preformed n3LCPUFAs and possibly due to an inhibitory effect of phenylalanine metabolites on the endogenous synthesis of docosahexaenoic acid (DHA) (Infante and Huszagh 2001). Suboptimal levels of n3LCPUFA may be related to neurologic (Agostoni et al. 2003) and bone (Lage et al. 2010) outcomes, and supplementation may improve subtle surrogate markers of neurologic function in patients with PKU (Gutierrez-Mata et al. 2012). There are several studies which suggest a possible benefit of n3LCPUFA supplementation on neurological outcomes in children with PKU. For example, fish oil supplementation in children with PKU has been shown to improve n3LCPUFA levels and motor skills (Beblo et al. 2007) and visual evoked potential latencies (Koletzko et al. 2009; Beblo et al. 2001). In a study of female patients over the age of 12, Yi et al. found a positive relationship between red blood cell (RBC) DHA levels and performance on a verbal ability task (Yi et al. 2011).

Unfortunately, most of the data available on n3LCPUFA status in PKU are for children. A recent meta-analysis on this topic (Lohner et al. 2013) reviewed 15 studies, only two of which included patients over the age of 25 (Lage et al. 2010; Moseley et al. 2002) for a total of 47 patients

over the age of 25 years. Despite the paucity of data on n3LCPUFA status in PKU adults, recent consensus guidelines (Singh et al. 2014) suggest that all patients given fat-free medical foods should have essential fatty acid status monitored. Some literature suggests that suboptimal n3LCPUFA status in adults may not be the same as seen in children (Moseley et al. 2002; Pöge et al. 1998), questioning the need for screening in adults and its associated costs. Recent work has also documented that adult patients with PKU have very limited access to expert care (Berry et al. 2013) so specialized laboratory testing may be even more restricted. Thus, it would be useful to identify standard nutrition screening tools to assess risk for suboptimal n3LCPUFA levels in order to reduce the need for specialized laboratory testing.

Methods

The University of British Columbia Clinical Research Ethics Board approved the study protocol and all subjects provided informed consent. Adults with PKU followed at our site and able to provide informed consent were invited to participate over a 12-month period. Plasma total fatty acid profiles are included in the VGH Adult Metabolic Clinic's annual laboratory assessment of PKU patients as this is a recommended component of the nutrition management for PKU (Singh et al. 2014). This blood work was sent to a commercial laboratory (Kennedy Krieger Institute, Baltimore, Maryland, USA), and other laboratory parameters including phenylalanine (PHE) levels were done locally using standard methods. The Kennedy Krieger laboratory has derived its normal range for plasma total fatty acids from 52 controls who reside in North America, but details of the ethnic makeup of the control population are not known (Dr. Richard Jones Ph.D., Administrative Laboratory Manager, Genetics Laboratory Kennedy Krieger Institute, personal communication, November 5, 2014). Data on docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), α-linolenic acid (ALA), and arachidonic acid (ARA) are included here. Levels within one standard deviation of the mean for laboratory controls were considered normal. Although the usual threshold for defining laboratory values as being "low" is −2 SD below the mean of healthy controls, we selected a higher threshold of −1 SD given our primary goal was to see if diet history could be used to define an at-risk population to allow appropriate targeting for n3LCPUFA screening. This lower threshold would reduce the chance that patients who might benefit from n3LCPUFA measurement and/or supplementation would be missed.

A nutrition history was obtained from each subject using the Omega-3 PUFA Food Frequency Questionnaire (Sublette et al. 2011) modified by asking additional questions about ALA, DHA, and EPA intake from PKU medical food products and adherence to the subjects' prescription of medical food products as shown in question 5 and 6 of the Appendix. The daily amount of n3LCPUFA consumed in the diet from food, supplements, and actual (not prescribed) formula intake was used based on these patient self-reports.

The normality of plasma level distributions was tested using the Shapiro–Wilk test. Student's *t*-test was used to compare data showing normal distribution, and the Mann–Whitney *U* test was used for data which were not normally distributed. Correlations were calculated with the Pearson correlation test. All conclusions were based on a significance level of $P < 0.05$.

Results

In a 12-month period, 53 adult subjects with PKU had plasma total fatty acid profiles drawn. Of these, 10 subjects were diagnosed with PKU before newborn screening and unable to provide informed consent, 1 was pregnant, 2 consented but did not have the blood work done, 2 declined consent, and 5 could not be contacted for consent so were excluded from analysis, leaving a total of 35 subjects for inclusion in the study. Clinical characteristics and laboratory parameters of the study participants are shown in Table 1.

Plasma DHA levels in study subjects were significantly lower ($p < 0.0001$) and plasma EPA ($p = 0.021$) and ALA ($p < 0.001$) significantly higher than laboratory normal controls. ARA levels in study subjects did not differ from control values ($p = 0.2$). Estimated dietary intake of DHA and EPA using the modified questionnaire correlated with plasma levels for DHA ($p < 0.001$) and for EPA ($p = 0.002$). Blood PHE levels did not correlate with DHA ($p = 0.74$), EPA ($p = 0.4$), ALA ($p = 0.2$), or ARA ($p = 0.1$) levels in study subjects.

Fifteen subjects had normal levels of DHA, and 14 of these subjects had normal levels of DHA, EPA, and ALA. Of the 15 subjects with normal levels of DHA, 10 were consuming DHA through supplements or a supplemented PKU medical food product. Three of the 5 subjects with normal DHA levels who did not obtain DHA from medical food products or supplements reported eating fish. There were two patients were on medical food product supplemented with DHA but had DHA levels <1 SD below the laboratory control range. Both subjects routinely consumed less than 50% of the DHA supplemented medical food product prescribed indicating limited compliance.

Predictive values were calculated from these data. If a subject was predicted on history to have adequate intake of DHA, then 93% of those subjects actually did have a

Table 1 Demographics, phenylalanine, and n3PUFA levels of adult subjects with PKU

Parameter	Subject values	Comment
N (male:female)	22:13	Data not available on sex of controls
Age (mean ± SD)	28.5 ± 7.5	
BMI (mean ± SD)	28.7 ± 6.6	
Blood PHE (μmol/L; median (range))	703 (range 103–1,721)	$N = 33$ as two subjects did not have PHE levels at the time of PUFA analysis
Subjects on supplemented formula	10	$N = 4$ on formula supplemented with ALA alone; $N = 5$ on formulas supplemented with DHA and EPA, and $N = 1$ on formula supplemented with DHA alone
DHA – (%total; mean ± SD)	1.65 ± 0.61	$p < 0.0001$ versus laboratory control range[a]
EPA – (%total; mean ± SD)	0.79 ± 0.31	$p = 0.021$ versus laboratory control range[a]
ALA – (%total; mean ± SD)	0.96 ± 0.32	$p < 0.001$ versus laboratory control range[a]
ARA – (%total; mean ± SD)	7.12 ± 1.67	$p = 0.2$ versus laboratory control range[a]

PHE phenylalanine, *n3LCPUFA* omega-3 polyunsaturated fatty acids, *DHA* docosahexaenoic acid, *EPA* eicosapentaenoic acid, *ALA* alpha-linoleic acid, *ARA* arachidonic acid

[a] *P* value compared with laboratory normal control values: control range was determined from 52 North American subjects aged (mean ± SD) 44 ± 13.8; range 18–75 years (Dr. Richard Jones Ph.D., Administrative Laboratory Manager, Genetics Laboratory Kennedy Krieger Institute, personal communication, November 5, 2014). The mean values (%total) of DHA, EPA, ALA, and ARA for the 52 controls used were 2.655%, 0.664%, 0.742%, and 6.751%, respectively, with a standard deviation of 0.968%, 0.366%, 0.307%, and 1.374%, respectively

normal plasma value of DHA, and measurement of n3LCPUFA levels in this group would be of low yield. If a subject was predicted on history to have low intake of DHA, then 90% of those subjects did have a low DHA and supplementation (with or without measurement of n3LCPUFA levels) could be considered on the basis of the diet history.

Discussion

Consistent with other authors, we have identified that levels of DHA are lower in PKU subjects although we did not identify deficiencies of other n3LCPUFA. Our intention, however, was to focus on findings useful from a patient management perspective. Firstly, information on n3LCPUFA intake obtained simply by diet history is sufficient to identify adult subjects at risk of suboptimal n3LCPUFA levels, suggesting this information can be used to target laboratory analysis or supplementation of n3LCPUFA levels only to those at risk.

Suboptimal DHA status is evident despite more than adequate levels of the precursor ALA. EPA levels were elevated, which differs from previous studies (Lohner et al. 2013), consistent with the findings of Infante and Huszagh (2001), suggesting that PHE metabolites may inhibit later stages in the pathway for DHA synthesis. This has relevance to current nutritional guidelines (Singh et al. 2014) which suggest that supplementation with the precursor ALA or with preformed DHA is appropriate in patients with low levels of n3LCPUFA. Our subjects have low levels of DHA despite elevated levels of ALA, suggesting that precursor supplementation would not be sufficient to normalize DHA levels in our adult population.

In our study of adult patients, we did not identify a relationship between blood PHE levels and n3LCPUFA status which is contrary to reports in children (Agostoni et al. 1997) and other reports in adults (Moseley et al. 2002). We are not sure of the reason for this discrepancy but speculate that it is related to the factors underlying poor control in our population. Dietary indiscretions in our patient population tend to be related to excessive intake of carbohydrate-rich foods, dairy, and meat rather than due to intake of n3LCPUFA protein sources such as fish, so noncompliance with dietary recommendations in our patients does not mean that their intake of DHA will increase.

Our study has limitations. Subject numbers are limited although this is the one of the largest data sets to date on n3LCPUFA levels of adult patients with PKU. We used lab-based normal values as a reference rather than our own controls. However, as the purpose of our study was not to repeat the work of other authors documenting n3LCPUFA deficiency in subjects with PKU but rather to identify ways we could target our screening to individuals at risk, we feel the use of lab-based normal ranges is reasonable. We also defined patients at risk of n3LCPUFA deficiency as being below 1 SD of the mean which is higher than the usually accepted thresholds of −2 SD of the mean. The predictive values of diet history in determining subjects at risk of

having low levels of n3LCPUFAs reported in this study are expected to change if the threshold to define a low level changes. However, in the absence of data which demonstrate clinical benefits of DHA supplementation in adults, we cannot comment on what threshold level of DHA is appropriate and choose a higher threshold to minimize the risk of failing to identify patients who might benefit from supplementation. Plasma levels of n3LCPUFA were used rather than measurement of tissue levels such as in RBC membranes which may be a more reliable measure of cellular availability of n3LCPUFA (Vilaseca et al. 2010). However, as assessment of membrane lipid content is not available clinically to most centers caring for adults with PKU (due to the practicality of sample handling in that samples have to be received at the lab within 24 h of collection), we feel the information on plasma levels is still useful to clinicians. Finally, geographic differences in dietary n3LCPUFA intake which have been described in other European countries (Acosta et al. 2001) may not make our results applicable to other centers.

Conclusions

Use of a modified diet history looking at sources of n3LCPUFA intake can be used to target screening of fatty acid levels to at-risk individuals with PKU. If supplementation is considered, preformed DHA may be the most appropriate form of supplementation. Further research is required to determine the optimal dose range for DHA supplementation and the effects of such supplementation, if any, on clinical outcomes in patients with PKU.

Compliance with Ethics Guidelines

Taryn Bosdet, Jennifer Branov, Caroline Selvage, Masoud Yousefi, and Sandra Sirrs declare that they have no conflict of interest.

Contributions of Individual Authors

Taryn Bosdet and Jennifer Branov were involved in the conception, design, conduct of the study, and the interpretation of data and drafting of article including revision for content of this manuscript.

Caroline Selvage's primary contribution was in the conduct of the study and data collection as well as the drafting of the manuscript.

Sandra Sirrs was involved in the analysis and interpretation of data as well as in the drafting and revision of the manuscript.

Masoud Yousefi performed analysis and interpretation of data and contributed to the drafting of the manuscript.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000(5). Informed consent was obtained from all patients being included in the study.

Appendix

Questions useful in assessing DHA intake and predicting DHA status (Questions 1–4 selected from Sublette et al. 2011):

1. How many times have you eaten fish or shellfish in any form?
   (1) Never
   (2) Less than 1 time each month
   (3) 1 time each month
   (4) 2–3 times each month
   (5) 1 time each week
   (6) 2 times each week
   (7) 3–4 times each week
   (8) 5–6 times each week
   (9) 1 time each day
   (10) 2 or more times each day
2. Each time you ate fish or shellfish, how much did you eat?
   (1) Less than 2 ounces or less than one fillet or less than 4 pieces of sushi
   (2) 2–7 ounces or about 1 fillet or 4–14 pieces of sushi
   (3) More than 7 ounces or more than 1 fillet or more than 14 pieces of sushi
3. In the past 6 months, about how often did you use cod liver oil?
   (1) Never
   (2) Less than 1 time each month
   (3) 1 time each month
   (4) 2–3 times each month
   (5) 1 time each week
   (6) 2 times each week
   (7) 3–4 times each week
   (8) 5–6 times each week
   (9) 1 time each day
   (10) 2 or more times each day

4. In the past 6 months, have you used an omega-3 fatty acid or fish oil supplement at least once each week?
   (1) No
   (2) Yes – What type of an omega-3 fatty acid or fish oil supplement did you take?
   Please write the name of the supplement below:
   (3) Is the omega-3 fatty acid or fish oil supplement in pill or capsule form?
      (1) No
      (2) Yes – How much did you take?
         (1) 1 pill or capsule each week
         (2) 2 pills or capsules each week
         (3) 3–4 pills or capsules each week
         (4) 5–6 pills or capsules each week
         (5) 1 pill or capsule each day
         (6) 2 pills or capsules each day
         (7) 3–4 pills or capsules each day
         (8) 5 or more pills or capsules each
   (4) Is the omega-3 fatty acid or fish oil supplement (besides cod liver oil) in liquid form?
      (1) No
      (2) Yes – How much did you take?
         (1) Less than 1 tablespoon each week
         (2) 1 tablespoon each week
         (3) 2 tablespoons each week
         (4) 3–4 tablespoons each week
         (5) 5–6 tablespoons each week
         (6) 1 tablespoon each day
         (7) 2 tablespoons each day
         (8) 3–4 tablespoons each day
         (9) 5 or more tablespoons each day
   (5) Please write down the dosage of omega-3 fatty acids or fish oil supplement if you know it:

      Dosage:
      Pills or Capsules: _____mg DHA and _____mg EPA per pill/capsule
      Liquid: _____mg DHA and _____mg EPA per tablespoon
      Do not know dosage

5. Do you take a PKU Medical Food Product?

   No
   Yes
   Please write down the name of your PKU Medical Food:

6. On average, how much of this PKU Medical Food do you take each day (please write down what you are actually taking, not what would be ideal):

   Scoops/packages: I take _____scoops/packages of formula each day
   Grams of powder: I take _____grams of powder per day
   Tetra's/pouches: I take _____pouches of formula per day

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CASE REPORT

Successful Pregnancy in a Woman with Maple Syrup Urine Disease: Case Report

Stefanie Heiber • Henryk Zulewski • Marianne Zaugg • Caroline Kiss • Matthias Baumgartner

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Abstract We present the positive outcome of a pregnancy in a woman with severe classic maple syrup urine disease (MSUD). Maintaining the maternal plasma levels of leucine between 200 and 300 μmol/L allowed normal development of the foetus. Tolerance of protein and leucine increased continuously from the 16th gestational week until delivery. The patient was able to increase protein and leucine intake from 5 g to nearly 30 g and 300–3,000 mg/day, respectively. Weekly measurement of branched-chain amino acid (BCAA) concentrations and the assessment of dietary intake were used to adjust protein intake. After 41 weeks of pregnancy, she gave birth to a healthy baby girl and was able to breastfeed her daughter for 6 months during which time, the protein and leucine intake were lower than during pregnancy, but higher than with her usual pre-pregnancy diet. The development of the girl is normal at the age of 3 years.

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Introduction

MSUD or branched-chain alpha-ketoaciduria is an autosomal recessive disorder of branched-chain amino acid (BCAA) catabolism first described in 1954 (Menkes et al. 1954). It is caused by a deficiency of branched-chain alpha-ketoacid dehydrogenase, which leads to an accumulation of the BCAAs leucine, isoleucine and valine and their toxic metabolites (Barbara 2010). Five distinct clinical phenotypes have been described in patients with MSUD, with 80% suffering from the severe classic type with 0–2% of normal enzyme activity (Chuang and Shin 2001). Untreated MSUD leads to severe mental and physical disabilities or death (Treacy et al. 1992). In 1964 administration of a diet restricted in BCAA was reported in seven patients with MSUD demonstrating that neurological manifestations could be prevented if nutritional management was instituted early (Snydermann et al. 1964), thereby establishing the basis for long-term nutritional management of patients with MSUD. In severe classical MSUD the nutritional therapy consists of strict protein restriction in combination with daily supplementation of a precursor-free amino acid mixture. Since then the outcome of patients with MSUD has gradually improved. Progress in newborn screening, early diagnosis and medical treatment, as well as an improved nutritional management, has resulted in normal growth and mental development in many of these patients (Hoffmann et al. 2006). As a result, an increasing number of women with MSUD reach child-bearing age. Since protein requirement increases during pregnancy, the management of such patients is a considerable challenge. Successful pregnancy outcomes in women with MSUD have been reported (Tchan et al. 2013; Grünewald et al. 1998; Van Calcar et al. 1992).

Case Report

The patient is a 31-year-old Caucasian woman who suffers from severe MSUD, diagnosed within the first week of life. She received dietary treatment with strictly reduced intake of protein and branched-chain amino acids. She only had one significant metabolic decompensation at the age of 18 months. Physical and mental development remained normal. Also during adolescence – often a difficult period to keep patients motivated to follow the diet – leucine levels were most of the time within the target range. Higher BCAA levels were usually attributed to short episodes of illness such as influenza. The normal mental development and her decision to become a nurse were surely helpful in comprehending the particular features of MSUD and were supportive for dietary compliance. The patient's usual diet in adulthood includes a very low protein diet with about 5 g natural protein per day derived from fruits and vegetables. This corresponds approximately to 300–500 mg leucine. To keep the protein and leucine intake that low and to reach an adequate energy intake, she consumes medical dietary products such as low protein pasta, bread, cereals or sweets. To ensure an appropriate protein intake, she daily takes 70 g of a BCAA-free, micronutrient-enriched amino acid mixture (ILV-AM®, SHS). She is aware of the importance of covering the energy requirements to avoid leucine increase from protein breakdown, i.e. she eats about five to six meals per day and she avoids catabolic fasting conditions. According to the regular laboratory results (leucine between 400 and 600 μmol/L), she consistently adhered well to her diet.

At the age of 29 she married an unrelated Caucasian man and became pregnant at the age of 30 years.

Pre-pregnancy anthropometric measures were height 153 cm, weight 58 kg and BMI 24.7 kg/m^2. Her calculated resting energy requirement was 1,350 kcal, total energy expenditure 2,000 kcal (38 kcal/kg), which corresponds to the recommended daily energy intake for adults with MSUD (Barbara 2010). Energy need for pregnancy was derived from requirements for healthy women plus approximately 250 kcal/day for the entire pregnancy. She presented in the seventh gestational week to the metabolic team. Based on the experience of the University of Wisconsin, Madison, United States, the goal was to keep plasma leucine concentration during pregnancy between 200 and 300 μmol/L which was slightly lower than usual. During the first 8 weeks, she followed her usual diet containing 300–500 mg leucine per day. First dietary counselling was set up after the first trimester of pregnancy. At this time the intake of leucine was around 1,000 mg/day. Plasma leucine concentrations were between 200 and 300 μmol/L, and she gained 1 kg in weight. She did not suffer from pregnancy sickness or vomiting.

Plasma leucine concentrations were measured weekly, and the patient maintained a food diary. Based on the plasma concentrations of BCAA, the intake of natural protein was increased. To keep plasma leucine concentrations in the target range, natural protein requirement continuously increased from the fourth month of gestation (Fig. 1). In a first step the low protein products were replaced by normal products. The daily intake of natural protein was increased to 15 g, corresponding to approximately 1,500 mg leucine, and the amino acid mixture was increased to 80 g. During the second half of pregnancy, a further increase to 30 g of natural protein was required to reach the target plasma BCAA values. This corresponded to approximately 3,000 mg leucine per day and was clearly much more than she had ever consumed before. In order to meet these increased protein needs, she had to consume more natural proteins. She disliked meat and fish, so that she ate more milk products and eggs. The maximal protein and leucine intake was in the eighth month of pregnancy. For a while she enjoyed this extraordinary situation; however, during the last month of pregnancy, she had no more appetite for those foods and she decreased the intake of natural protein to approximately 20 g/day. Despite this, the BCAA levels were maintained within the defined range (Fig. 2). During the whole pregnancy weight gain was about 10 kg.

After 41 weeks of gestation our patient gave birth to a healthy baby girl (weight 3,430 g, height 48 cm, head circumference 33.5 cm). To avoid catabolism during labour, she received intravenous glucose (220 g/24 h) from the moment the birth process started. The infusion was continued for the next 2 days. The maternal BCAA levels 5 h after birth were valine 205 μmol/L, isoleucine 63 μmol/L and leucine 174 μmol/L. The baby's BCAA levels were checked on day two after birth and were below the normal range: isoleucine 14.9 μmol/L, leucine 43.8 μmol/L and valine 85.2 μmol/L. For the first few days after delivery, the mother followed her pre-pregnancy diet with about 500 mg leucine per day. The postpartum goal for the BCAA levels of the mother was the same as those before pregnancy. Breastfeeding was instituted successfully at day two after birth. It was possible to discharge mother and baby after 5 days. The patient continued breastfeeding for the next 6 months. The BCAA levels were controlled approximately every third week. Three weeks after birth we found rather low levels of leucine (118 μmol/L) and valine (148 μmol/L). At this point the mother's intake of natural protein was 5–10 g/day. Based on the measured levels, the protein intake was increased to almost 15 g/day, and this was the average intake of natural protein per day during breastfeeding period. After stopping breastfeeding, she returned to her usual MSUD diet. The baby thrived very well, and at the age of 3 years she appeared to be normal in all aspects. The parents are planning a further pregnancy.

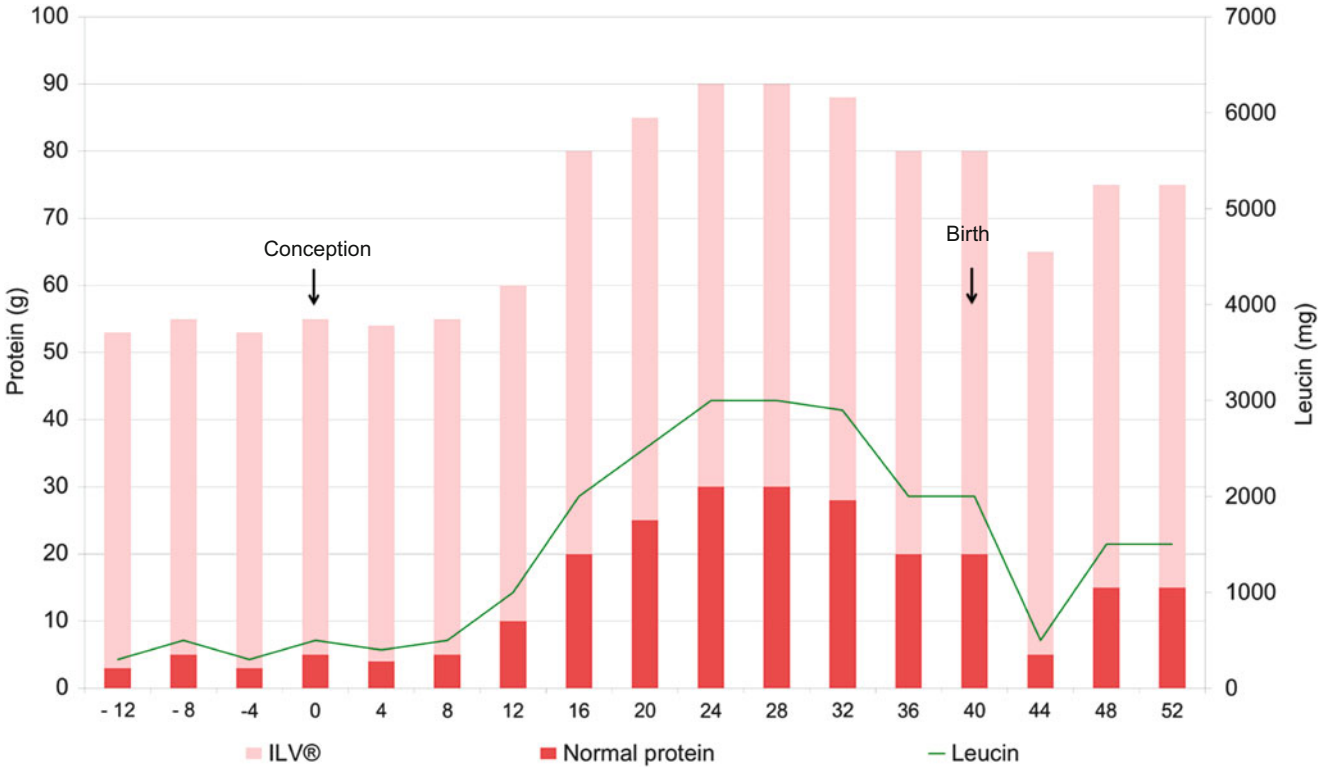

Fig. 1 Intake of total protein, aminoacid-mixture and normal protein (g). Intake of leucine (mg)

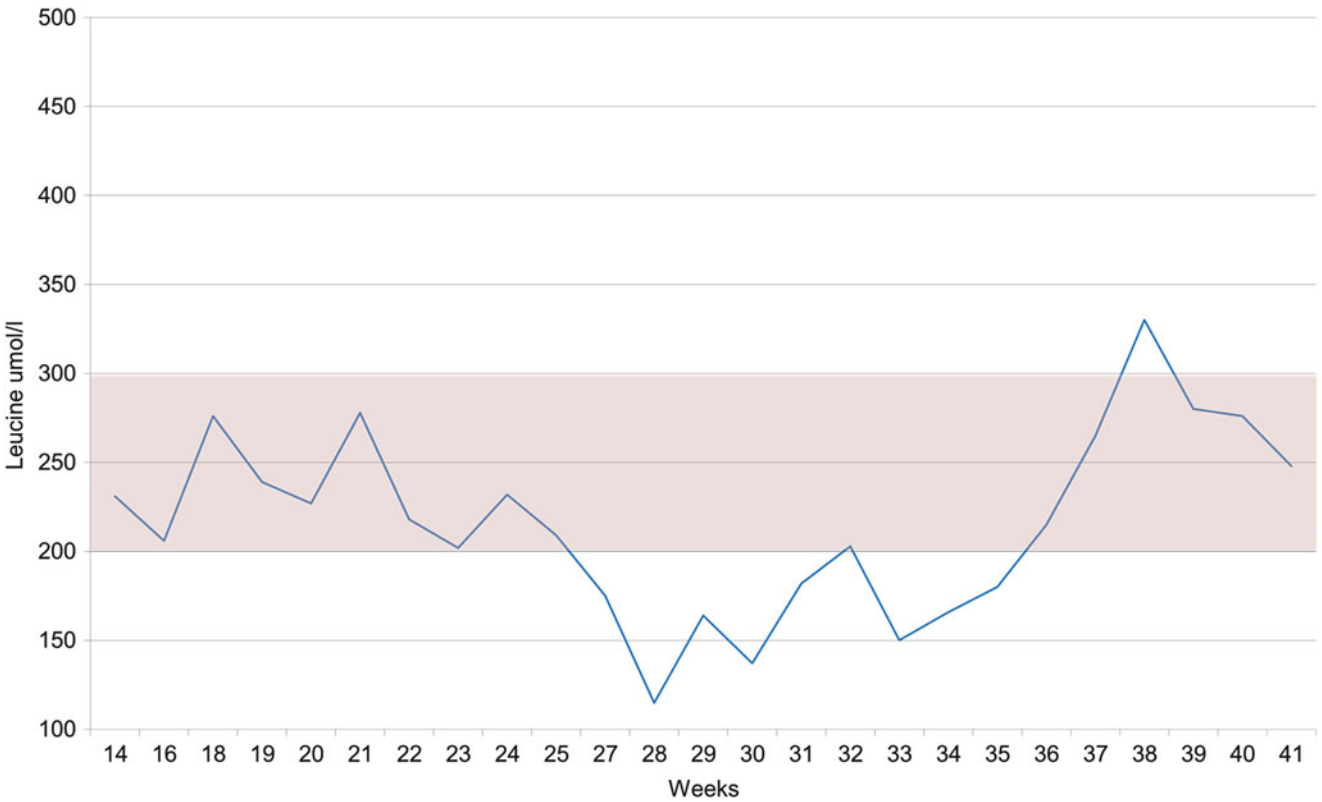

Fig. 2 Plasma leucine concentration (μmol/l) during pregnancy

Discussion

This is the sixth case of a successful pregnancy in a woman with classical MSUD documented in the literature (Tchan et al. 2013; Grünewald et al. 1998; Van Calcar et al. 1992). Remarkable in our case is the patient's excellent compliance with the diet. In order to maintain her compliance and motivation, we involved her in every possible process. This included the calculation of protein requirements and leucine intake as well as frequent regular follow-up visits with the metabolic team (physician, dietician and laboratory staff).

Successful outcomes of pregnancies in MSUD patients were presented by Tchan et al. (2013), Grünewald et al. (1998), and Van Calcar et al. (1992). Compared to the cases of Van Calcar and Tchan, our patient had a much lower pre-pregnancy protein intake (29, 15–35 and 30–50 g, respectively, versus 5 g natural protein in our patient). This was because of higher leucine tolerance or suboptimal compliance of the patient(s). While Van Calcar found a very high leucine tolerance of 8,600 mg/day at the end of the pregnancy suggesting a mild form of MSUD, Grünewald's patient's highest leucine tolerance (2,100 mg leucine per day) just before giving birth in the 36th week was closer to that to our patient. We found a progressive increase of leucine tolerance up to 3,000 mg of leucine per day and a maximal natural protein intake of approximately 30 g/day around week 30. As with Grünewald et al., we assume the increased tolerance of natural protein during pregnancy reflects enhanced protein synthesis in the fetomaternal compartment because of the capacity for branched-chain amino acid metabolism in the foetus that partially compensated the mother's metabolic deficit. In contrast to other cases, we found no increase in leucine level postpartum. Those high leucine levels were thought to be the result of protein breakdown due to postpartum uterine involution and perhaps also poor dietary compliance (Tchan). In order to avoid a postpartum rise of leucine, we therefore decided to attempt to avoid the catabolic state during labour and birth by giving an infusion of 10% glucose (3.3 g/kg/day). We also reduced leucine intake to pre-pregnancy values immediately postpartum until breastfeeding was successfully instituted. Since one hundred ml of mother's milk contains about 1.5 g protein and 300 mg leucine, we expected an increase of protein and leucine requirement during the breastfeeding period.

Our case and the described examples clearly demonstrate that careful and close monitoring of MSUD patients is very important during pregnancy and the experience gained is helpful to handle further cases. Although it is clear that it remains essential to treat every case individually, this needs to be based on the careful and detailed documentation of management and outcome of pregnancies in women with MSUD as reported here. Finally a metabolic team with experience of patients with inherited metabolic diseases, with close collaboration of physicians, laboratory staff, dietician and the patient, is essential for successful outcome in pregnancy of MSUD and related disorders.

Compliance with Ethics Guidelines

Informed Consent: The patient was informed and agreed to the use of her anonymised data for the purpose of this publication.

Conflict of Interest

Stefanie Heiber, Henryk Zulewski, Marianne Zaugg, Caroline Kiss and Matthias Baumgartner declare that they have no conflict of interest.

Author Contributions

Stefanie Heiber – Dietician counselling, adapted dietary recommendations based on laboratory findings. Wrote the manuscript, guarantor

Henryk Zulewski – Followed the patient before, during and after pregnancy, discussed the manuscript

Marianne Zaugg – Laboratory investigation, interpretation of laboratory findings, discussed the manuscript

Caroline Kiss – Discussed the manuscript

Matthias Baumgartner – Followed the patient before, during and after pregnancy, discussed the manuscript

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RESEARCH REPORT

Hepatic Copper Accumulation: A Novel Feature in Transient Infantile Liver Failure Due to *TRMU* Mutations?

Z. Grover • P. Lewindon • A. Clousten • A. Shaag • O. Elpeleg • D. Coman

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Abstract Defects in the mitochondrial respiratory chain can induce a heterogeneous range of clinical and biochemical manifestations. Hepatic involvement includes acute fulminant hepatic failure, microvesicular steatosis, neonatal non-alloimmune haemochromatosis and cirrhosis. Recently pathogenic mutations in tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase (*TRMU*) gene (OMIM 610230) have been demonstrated to cause transient infantile liver failure (OMIM 613070). The human *TRMU* gene encodes a mitochondrial protein, 5-methylaminomethyl-2-thiouridylate methyltransferase, whose molecular function is that of mitochondrial tRNA modification.

We report an infant who presented with acute liver failure, in whom we observed hepatic copper intoxication and cirrhosis on liver biopsy. We postulate that the hepatic copper intoxication observed in our patient is most likely a secondary event associated with cholangiopathy. Periportal copper accumulation has been implicated in causing secondary mitochondrial dysfunction; the impact of copper accumulation in patients with *TRMU* mutations is unclear and warrants long-term clinical follow-up.

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Introduction

The mitochondrial respiratory chain (MRC) is an essential component of normal cellular activity. Mitochondria are double-membraned intracellular organelles whose main function is to generate the high-energy phosphate molecule ATP via the process of oxidative phosphorylation (OXPHOS) (Lee and Sokol 2007). In order to achieve OXPHOS, the MRC is constructed into five separate complexes in which the subcomponents are encoded by both nuclear and mitochondrial DNA (mtDNA), with Complex II being the only complex without mtDNA contributions to its structure or maintenance.

The clinical features of a mitochondrial disorder are protean, in which "high-energy requirement" organs are more likely to produce a measurable disease burden. Hepatic involvement can occur as part of a single-organ or multi-organ clinical phenotype. The hepatic involvement of MRC disorders is common and demonstrates heterogeneity in clinical presentations and age of onset and is often fatal especially in the neonatal-infantile period. The hepatic features most commonly associated with MRC diseases include cholestasis, coagulopathy, cirrhosis, non-alloimmune neonatal haemochromatosis and acute fulminant hepatic failure (Lee and Sokol 2007; Fellman and Kotarsky 2011). Liver biopsy most commonly demonstrates hepatic steatosis.

In recent years nuclear-encoded genes that play a role in the maintenance and translational efficiency of mtDNA have been linked with hepatic phenotypes due to mitochondrial depletion. Nuclear-encoded genes associated with mitochondrial depletion syndromes include *POLG* (OMIM 174736), *MPV17* (OMIM 137960), *SUCLG1* (OMIM 611224) and *DGUOK* (OMIM 601465) (Fellman and Kotarsky 2011). Mutations in the tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase (*TRMU*) gene (OMIM 610230) have recently emerged as a cause of infantile-onset acute hepatic failure (Zeharia et al. 2009). The *TRMU* gene product functions as a mitochondrial tRNA modification and thus mitochondrial translation (Sasarman et al. 2011). An encouraging feature of this clinical presentation is that the hepatic failure can be reversible with appropriate supportive treatment. However, long-term neurological and hepatic outcomes are yet to be defined. Our case differs to the reported cases with *TRMU* mutations in that periportal hepatocyte copper loading was evident on liver biopsy.

Case Report

Our previously well female infant presented in extremis in hypovolaemic shock with acute hepatic failure at 4.5 months of age. There was no obvious precipitating infective illness. She had a gastrointestinal haemorrhage secondary to severe coagulopathy and was found to be profoundly hypoglycaemic and acidotic from a high lactate. She ultimately made a full systemic, hepatic and neurological recovery with intensive respiratory, circulatory and liver supportive therapy (*N*-acetylcysteine, carnitine and coenzyme Q10).

A primary mitochondrial defect was considered as part of the differential diagnosis in view of the abruptness and severity of clinical and biochemical presentation. Persistent mild abnormalities in liver function combined with the absence of an alternative diagnosis prompted the collection of liver and muscle biopsies at 8 months of age.

Significant abnormalities were detected on histological examination of the liver biopsy, most notably copper loading. Copper staining with rhodanine was performed revealing conspicuous copper and copper binding protein deposition in hepatocytes in a periportal distribution with no evidence of Mallory hyaline deposits (Fig. 1). Other prominent histological findings included portal tract fibrosis, cholangiopathy and small droplet steatosis (Figs. 2 and 3). Electron microscopy of the liver tissue revealed excessive numbers of mitochondria per cell, providing ultrastructural morphology correlation of oncocytosis noted on light microscopy with microvesicular steatosis and normal mitochondrial morphology.

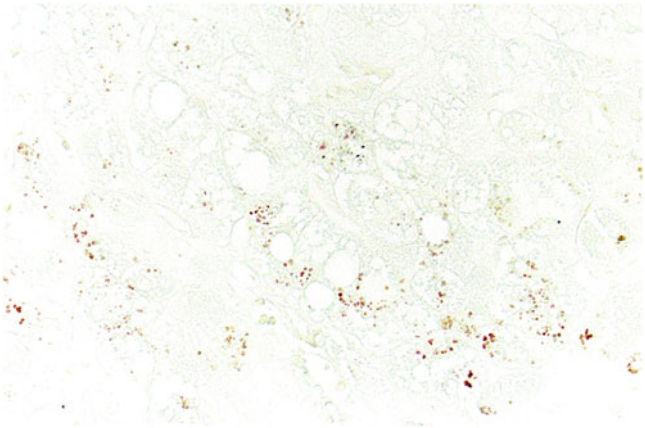

Fig. 1 Rhodanine staining revealing conspicuous copper and copper binding protein deposition in hepatocytes in a periportal distribution with no evidence of Mallory hyaline deposits

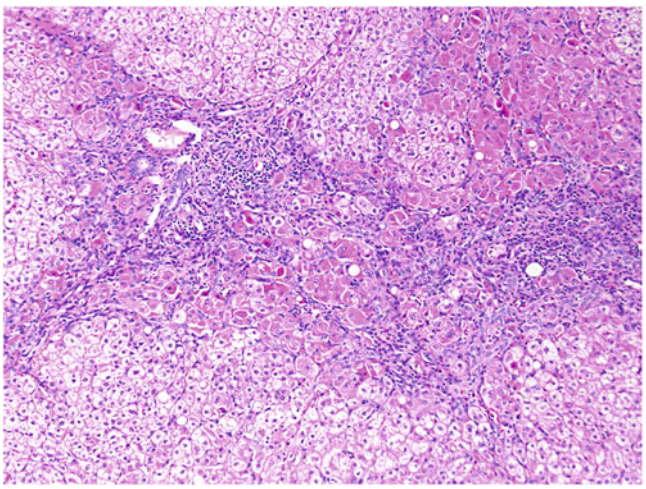

Fig. 2 Portal tract fibrosis, cholangiopathy and small droplet steatosis

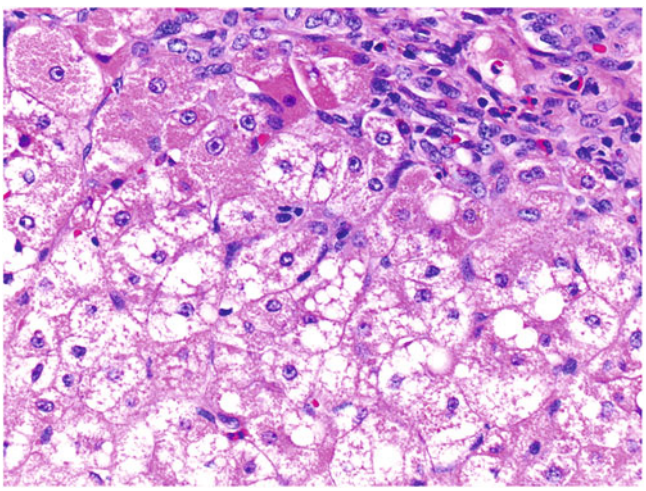

Fig. 3 Portal tract fibrosis, cholangiopathy and small droplet steatosis

Table 1 Mitochondrial respiratory chain enzymes – liver

Unit (nmol/min/mg)	Activity (ref range)	% Activity	% CS ratio	% CII ratio
Complex I	12 (8–11)	126	29	58
Complex II	134 (54–73)	220	49	
Complex III	9.5(5.2–10.3)	125	28	57
Complex IV	0.45 (0.5–0.9)	63	14	29
Citrate synthase	122 (26–31)	436		

The patient had no dietary history to suggest excess copper ingestion and no wider clinical features associated with known genetic defects in copper transportation. Serum copper levels (16 μmol/L, reference range 13–26) and ceruloplasmin levels (1.75 μmol/L, reference range 1.9–3.41) were within normal limits.

Mitochondrial respiratory chain (MRC) enzyme analysis occurred via previously published methods (Bernier et al. 2002) with normal results in the muscle. Liver MRC chain activity levels of Complexes I, III and IV were low compared to citrate synthase, and Complex IV activity was low compared to Complex II activity (see Table 1). Mutation analysis for the common mitochondrial DNA mutations and the three common *POLG* mutations yielded normal results. The hepatic mtDNA/nDNA ratio was 1.02. *TRMU* sequencing proceeded via previously published methods (Zeharia et al. 2009) identifying compound heterozygosity for two pathogenic mutations c.835G>A and c.1037-1040 del TCAA.

Having identified periportal copper staining and hepatic fibrosis, we instituted a hepatocellular carcinoma surveillance programme involving yearly liver ultrasounds and plasma alpha-fetoprotein levels. In the absence of a formal diagnosis of Wilson's disease, specific copper chelation was avoided, but it was thought prudent to commence zinc acetate to reduce enterocyte absorption of copper. Now 4 years later our patient continues to demonstrate normal neurological developmental and hepatic function. Liver synthetic function and transaminases, copper studies and regular hepatobiliary ultrasound scanning are normal.

Discussion

The aetiology of acute infantile hepatic failure is heterogeneous in nature, and mitochondrial respiratory chain defects are potentially under-recognised. Diagnosis is confounded by non-specific clinical phenotypes, technical difficulties in obtaining and interpreting mitochondrial respiratory chain studies and molecular heterogeneity of mitochondrial defects. An accurate and timely diagnosis for the hepatic failure and of the molecular aetiology of mitochondrial respiratory chain defects is of significant practical importance for the hepatologist in terms of liver transplantation suitability. The reversible hepatic failure seen in patients with *TRMU* mutations underscores the importance of making this diagnosis and the value of aggressive supportive care. The clinical presentation and recovery of our infant are similar to reported cases associated with *TRMU* mutations (Gaignard et al. 2013; Schara et al. 2011; Uusimaa et al. 2011; Zeharia et al. 2009). Our infant presented with acute hepatic failure, had mild deficiency of MRC Complex IV on liver samples and has made a full and sustained clinical recovery. This case is unique in that the histopathological findings revealed excessive periportal copper deposition in the liver biopsy.

Copper is an essential trace element, and both excess and deficiency are associated with clinically relevant pathology. Once dietary copper is absorbed from the enterocyte, it is transported to the liver via the portal venous system bound to amino acids like histidine and serum proteins like albumin. Delivery of copper into the hepatocyte occurs via the copper transport protein, and once into the hepatocyte, most copper is bound to caeruloplasmin, the major transport protein for copper. Within the liver, there are four potential routes for copper distribution, (1) joining the copper/metallothionein pool, (2) binding to copper chaperone protein for delivery to zinc superoxide mutase, (3) attaching to cox17 for trafficking into the mitochondria for MRC Complex IV assembly and (4) trafficking to the trans-Golgi network (TGN) via human atox-1 homologue (Shim and Harris 2003). Copper plays an essential role in numerous crucial enzymatic pathways including neurotransmitter production, lysyl oxidase cross-linkage of collagen, free radical savaging via superoxide dismutase and metallothioneins and formation of MRC Complex IV and clotting factors (Corkins 2011). Genetic diseases of copper excretion and intracellular transport are associated with varied clinical phenotypes. These genetic diseases are associated with the TGN transportation of copper and are Menkes disease (MD) (OMIM 309400) and Wilson's disease (WD) (OMIM 277900).

Under normal physiologic conditions 80% of copper is excreted via the biliary system attached to bile acids and

20% via the urine (Corkins 2011). Diseases associated with copper excretion are caused by a primary genetic defect in the excretion pathway, such as those associated with the TGN or by the process of biliary obstruction (Corkins 2011; Elmes et al. 1989; Evans et al. 1980; Goldfischer et al. 1980; Prohaska 2008). The periportal location of copper staining in our patient is more in keeping with being secondary to altered biliary excretion rather than from dietary copper excess as seen in Indian childhood cirrhosis (Pankit and Bhave 2002), endemic Tyrolean infantile cirrhosis (Pankit and Bhave 2002) or a TGN-related copper transport defect in which the copper distribution tends to be pan-lobar (Goldfischer et al. 1980).

Our case raises unanswered questions regarding the role of copper in the pathogenicity of liver failure secondary to patients with *TRMU* mutations. The most likely reason for the periportal copper staining in our patient is secondary to abrupt biliary cholestasis secondary to severe mitochondrial impairment. To our knowledge, the only reported functions of the *TRMU* gene product have been of mitochondrial 2-thiolation of the wobble U in tRNAlys, tRNAGlu and tRNAGln (Sasarman et al. 2011). A cytosolic role, especially one associated with intracellular copper trafficking, has not been postulated. MRC Complex IV is a critical component of the OXPHOS pathway in which the catalytic core contains three copper atoms (Horn and Barrientos 2008; Mehta et al. 2006). The *TRMU* gene product has not been postulated to have a role as a cytosolic or an intramitochondrial copper chaperone molecule or in that of MRC Complex IV assembly. However, MRC Complex IV deficiency has been described in patients with *TRMU* mutations including our case (Gaignard et al. 2013; Schara et al. 2011; Uusimaa et al. 2011; Zeharia et al. 2009). Hepatic copper deposition does not equate to systemic copper excess, as demonstrated in our patient. Cases of symptomatic copper deficiency have been described in patients with chronic cholestasis when copper has been removed from total parenteral nutrition supplements (Corkins 2011), highlighting the importance of copper for normal cellular function.

Oxidative stress is implicated in the pathogenesis and progression of specific liver diseases including biliary cirrhosis (Sastre et al. 2007; Tiao et al. 2009). In chronic cholestasis liver mitochondria have demonstrated increased H_2O_2 production and GSH depletion and oxidation (Sastre et al. 2007). Mitochondrial oxidative stress is a precursor to apoptosis. Copper excretion from the TGN involves interaction between MURR1/*COMMD1* and the X-linked inhibitor of apoptosis protein (XIAP), which is a potent suppressor of apoptosis that directly inhibits specific members of the caspase family of cysteine proteases (Burstein et al. 2004). XIAP levels are greatly reduced by intracellular copper accumulation in Wilson's disease and other copper toxicosis disorders (Mufti et al. 2006, 2007). Elevated copper levels result in a profound, reversible conformational change in XIAP, which accelerates degradation and significantly decreases the ability of XIAP to inhibit caspase-3 (Mufti et al. 2006, 2007). The observation of periportal copper accumulation in our patient is likely to be secondary to cholestasis; however, copper accumulation has been associated with the initiation of apoptosis via XIAP and mitochondrial oxidative stress. The *TRMU* gene product functions in the mitochondrial matrix; what is unclear from our case is if the copper accumulation will induce long-term secondary mitochondrial oxidative stress in an already abnormal mitochondrial system.

In conclusion, this case highlights the importance of recognising *TRMU* mutations as a cause of reversible, transient liver failure in infants and provides some insight into the potential interaction of mitochondrial disorders and intrahepatic copper accumulation. As the long-term outcome from liver failure in infants with *TRMU* mutations, in particular those with copper accumulation, has yet to be defined, our case also highlights the need for long-term follow-up of these patients.

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Compliance with Ethics Guidelines

Conflict of Interest

Zubin Grover, Pete Lewindon, Andrew Clousten, Avraham Shaag, Orly Elpeleg and David Coman declare that they have no conflicts of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

Author Contributions

Dr. Zubin Grover was a paediatric gastroenterologist involved in patient care and the manuscript development.

A/Prof. Lewindon was a paediatric gastroenterologist involved in patient care and the manuscript development.

Dr. Andrew Clousten is a histopathologist who reports the liver biopsy histology and has been involved in the manuscript development.

Drs. Orly Elpeleg and Avrahm Shaag performed the TRMU gene sequencing and assisted in the manuscript development.

A/Prof. David Coman is a metabolic physician and the patient's primary care giver and has coordinated the manuscript development and design as the senior author.

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CASE REPORT

Infantile Cases of Sitosterolaemia with Novel Mutations in the ABCG5 Gene: Extreme Hypercholesterolaemia is Exacerbated by Breastfeeding

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Abstract Few data exists regarding the clinical impact of breastfeeding in infantile sitosterolaemic cases. We report four Japanese infantile cases of sitosterolaemia, an extremely rare inherited disease characterised by increased serum levels of plant sitosterol, presenting with severe hypercholesterolaemia and systemic xanthomas exacerbated by breastfeeding. In these four cases, genetic analyses were performed for low-density lipoprotein (LDL) receptor, proprotein convertase subtilisin/kexin type 9 (PCSK9), LDL receptor adaptor protein 1 and ATP-binding cassette (ABC) subfamily G member 5 and 8 (ABCG5 and ABCG8) genes. We assessed their clinical manifestations, including responsiveness to a variety of treatments, especially to weaning from breastfeeding and use of ezetimibe. Two pairs of mutations in the ABCG5 gene in each case, including two novel mutations (c.130C>T or p.Ser44Ala and c.1813_1817delCTTTT or p.Pro558GlufsX14) and two known mutations (c.1306G>A or p.Arg389His and c.1336C>T or p.Arg446X), were identified. Significant reductions in cholesterol levels were obtained by means of weaning from breastfeeding alone. Substantial reductions in sitosterol levels, without any apparent side effects, were observed with ezetimibe. In conclusion, we have identified infantile Japanese sitosterolaemic subjects with extreme hypercholesterolaemia exacerbated by breastfeeding. Their unique response to weaning from breastfeeding, as well as to use of ezetimibe, could provide insights into the metabolic basis of sterols in humans.

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Introduction

Sitosterolaemia (OMIM #210250) caused by mutations in either of two genes, ATP-binding cassette (ABC) subfamily G members 5 and 8 (ABCG5 and ABCG8), is an extremely rare autosomal recessive disorder of sterol metabolism characterised by increased absorption and decreased biliary excretion of plant sterols and cholesterol, resulting in prominently elevated serum levels of plant sterols such as sitosterol and campesterol (Shulman et al. 1976). Subjects suffering from sitosterolaemia present primarily with tendinous and tuberous xanthomas and premature coronary atherosclerosis resembling familial hypercholesterolaemia (FH: Salen et al. (2002), Bhattacharyya and Connor (1974)). Serum LDL cholesterol (LDL-C) levels tend to vary more in sitosterolaemia than in other genetic or nongenetic hyperlipidaemias. Notably, LDL-C can be elevated significantly in some cases with sitosterolaemia, especially in infantile cases, due to unknown mechanisms (Yoshida et al. 2000). Thus, those cases are sometimes misdiagnosed as homozygous FH.

We encountered four Japanese infantile cases initially diagnosed as homozygous FH based on their clinical manifestations of severe hypercholesterolaemia and systemic intertriginous xanthomas. However, those manifestations responded very well to a variety of treatments, especially weaning from breastfeeding, which would not be observed in homozygous FH. Therefore, the aims of this study were (1) determining their molecular diagnosis, including LDL receptor, proprotein convertase subtilisin/kexin type 9 (PCSK9) and LDL receptor adaptor protein 1 (LDLRAP1) genes that have been described as causative genes of Japanese FH (Mabuchi et al. 2014) as well as ABCG5/8 genes as causes of Japanese sitosterolaemia (Tsubakio-Yamamoto et al. 2010), and (2) evaluating their responsiveness to a variety of treatments.

Materials and Methods

Study Subjects

The backgrounds of the study subjects are listed in Supplemental Tables 1, 2, and 3. All of the probands showed severe hypercholesterolaemia, and three of them exhibited intertriginous xanthomas, but no subjects showed tendon xanthomas. None of them were born from consanguineous marriages, and none of their parents exhibited any sign of physical xanthomas. In all the study subjects, we noted no evidence of secondary hypercholesterolaemia, such as thyroid insufficiency or nephritic syndrome. The families initially visited three different institutions (Kanazawa University Hospital, Gifu Prefectural General Medical Center and Miyazaki University Hospital). And the families from Gifu Prefectural General Medical Center and Miyazaki University Hospital were referred to Kanazawa University Hospital because of the (initial) clinical diagnosis of homozygous FH.

Ethical Considerations

This study was approved by the Ethics Committee of Kanazawa University and carried out in accordance with the Declaration of Helsinki (2008) of the World Medical Association. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Informed consents were obtained from all subjects or their parents (if the subject was an infant) when they visited Kanazawa University Hospital for being included in the study.

Genetic Studies

Genomic DNA was isolated from peripheral white blood cells according to standard procedures and was used for polymerase chain reaction (PCR). Primers for the study were designed as previously reported (Noguchi et al. 2010); PCR products were purified by Microcon (Millipore Corp., Bedford, MA) and used as templates for direct sequencing. DNA sequencing was carried out according to the manufacturer's instructions, using a dye terminator method, ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). We analysed ABCG5/ABCG8 genes as well as LDL receptor, PCSK9 and LDLRAP1 genes, as previously reported (Mabuchi et al. 2014). In order to precisely determine the deleted codons in Case 1, Case 2 and Case 4, we digested the PCR products using DraI, which could recognise the 6-bp nucleotide TTTAAA and consequently make a 28-bp and an 85-bp fragment in the normal allele. Thus, we could discriminate the normal and abnormal alleles in exon 12 of the ABCG5 gene after electrophoresis through 2.5% polyacrylamide gels.

Biochemical Analysis

Blood samples were drawn for assays after overnight fasting. Serum levels of total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C) were determined enzymatically (Allain et al. 1974; Sugiura et al. 1977; Kajikawa et al. 1981). Serum levels of LDL-C were derived from the Friedewald's formula (Friedewald

et al. 1972). Serum cholesteryl ester transfer protein (CETP) levels were determined by a specific enzyme-linked immunosorbent assay (Kiyohara et al. 1998). Serum levels of sterol, including those of sitosterol, lathosterol and campesterol, were determined using gas–liquid chromatography–mass spectrometry (Ahmida et al. 2006).

Results

Genetic Analysis (Fig. 1, Supplemental Fig. 1)

No mutation was identified in the three FH genes (LDL receptor, PCSK9 and LDLRAP1) amongst any of the four sitosterolaemic cases.

In Case 1, direct sequencing analysis for exon 12 of ABCG5 gene showed abnormal overlapping after nucleotide position 1813, suggesting deletion of several nucleotides in this region (Fig. 1a). To clarify the supposed overlapping sequence, we attempted to digest the PCR products with DraI and found no fragmentation of PCR products that would have suggested the existence of an abnormal sequence (Fig. 1b). After direct sequencing of the non-fragmented PCR products, we could confirm a novel mutation of a 5-nucleotide deletion (c.1813_1817delCTTTT or p.Pro558GlufsX14) causing a premature termination at codon 571 (Fig. 1c, d). In addition, we identified another mutation in exon 9 (c.1306G>A or p.Arg389His, Fig. 1e), which is one of the most common mutations in Asian sitosterolaemic cases (Park et al. 2014). We confirmed the same mutations in Case 2 (the younger sister of Case 1). In Case 3, another novel substitution mutation in exon 1 of the ABCG5 gene (c.130T>C or p.Ser44Ala, Fig. 1f) and the same mutation in exon 9 as observed in Cases 1 and 2 (c.1306G>A or p.Arg389His, Fig. 1e) were identified by direct sequencing analysis, despite no apparent evidence of a familial relationship with Case 1 and Case 2. In Case 4, the same deletion mutation (c.1813_1817delCTTTT or p.Pro558GlufsX14) was detected in the ABCG5 gene, as observed in Case 1, despite no apparent evidence of a familial relationship with Case 1. In addition, another mutation was found in exon 10 of ABCG5 (c.1336T>C or p.Arg446X, Fig. 1g). The latter is also one of the most common mutations in Asian sitosterolaemic cases (Wang et al. 2014). No other mutation was found in either the ABCG5 or the ABCG8 gene in any of the four cases.

Clinical Courses and Interventions (Fig. 2)

The decisions on the treatments of the four cases were made based on their personalities, the policies of each institution and the preferences of the parents, providing us an opportunity to observe responsiveness to a variety of treatments.

The original serum TC, LDL-C and sitosterol levels of Case 1 were 523 mg/dl, 407 mg/dl and 101 μg/ml, respectively. These levels were reduced to 151 mg/dl, 81 mg/dl and 46 μg/ml, respectively, with 10 mg ezetimibe daily (gradually increased from 2 mg daily) and a low-cholesterol (<200 mg daily) and low-plant-sterol diet (avoiding vegetable oils, nuts and cereals), after weaning (Fig. 2a). Intertriginous xanthomas (Supplemental Fig. 2a, b) completely regressed in association with the reduction of these sterol levels (Supplemental Fig. 2c, d).

In the initial blood test, the serum levels of sitosterol in Case 2 were lower than those of Case 1 (Case 2's elder sister), whereas the serum levels of TC were similar for Cases 1 and 2. Weaning from breastfeeding was effective in reducing the TC levels of Case 2, but it increased her sitosterol levels; this increase was probably also associated with increased sitosterol intake. However, additional treatment with ezetimibe further reduced her TC and sitosterol levels (Fig. 2b). In this case, ezetimibe was introduced at 10 mg daily because this dose was well tolerated in Case 1 (Case 2's elder sister). In Case 3, the original serum TC, LDL-C and sitosterol levels were as high as 870 mg/dl, 796 mg/dl and 80 μg/ml, respectively. In a clinical course similar to that of Case 1, these levels dramatically reduced to 228 mg/dl, 157 mg/dl and 64.5 μg/ml, respectively, with administration of 10 mg of ezetimibe daily and a low-cholesterol (<200 mg daily) and low-plant-sterol diet (avoiding vegetable oils, nuts and cereals) after weaning (Fig. 2c). Ezetimibe was introduced at 10 mg daily in this case, based on discussions with her parents. Her intertriginous xanthomas (Supplemental Fig. 3) also successfully regressed (data not shown). In a similar way to that of Cases 2 and 3, in Case 4, weaning itself resulted in a great reduction in serum TC and LDL-C levels (from 756 mg/dl and 589 mg/dl to 326 mg/dl and 115 μg/ml, respectively) (Fig. 2d). Interestingly, his intertriginous xanthomas (Supplemental Fig. 4) completely disappeared at the age of five (data not shown). Ezetimibe at 10 mg daily, rather than colestimide, was effective in reducing serum sitosterol levels.

It is noteworthy that whilst weaning itself was effective in reducing TC and LDL-C levels and in promoting the regression of intertriginous xanthomas, it was not sufficient to reduce serum sitosterol levels (Table 1). Although the impact on the degree of reduction was different in each case, ezetimibe was effective in reducing serum sitosterol levels in all four cases.

Although several papers have suggested the association between sitosterolaemia and haematologic abnormalities, such as macrothrombocytopaenia, haemolytic anaemia and splenomegaly (Park et al. 2014; Wang et al. 2014), none of our cases exhibited any abnormal haematological manifestations.

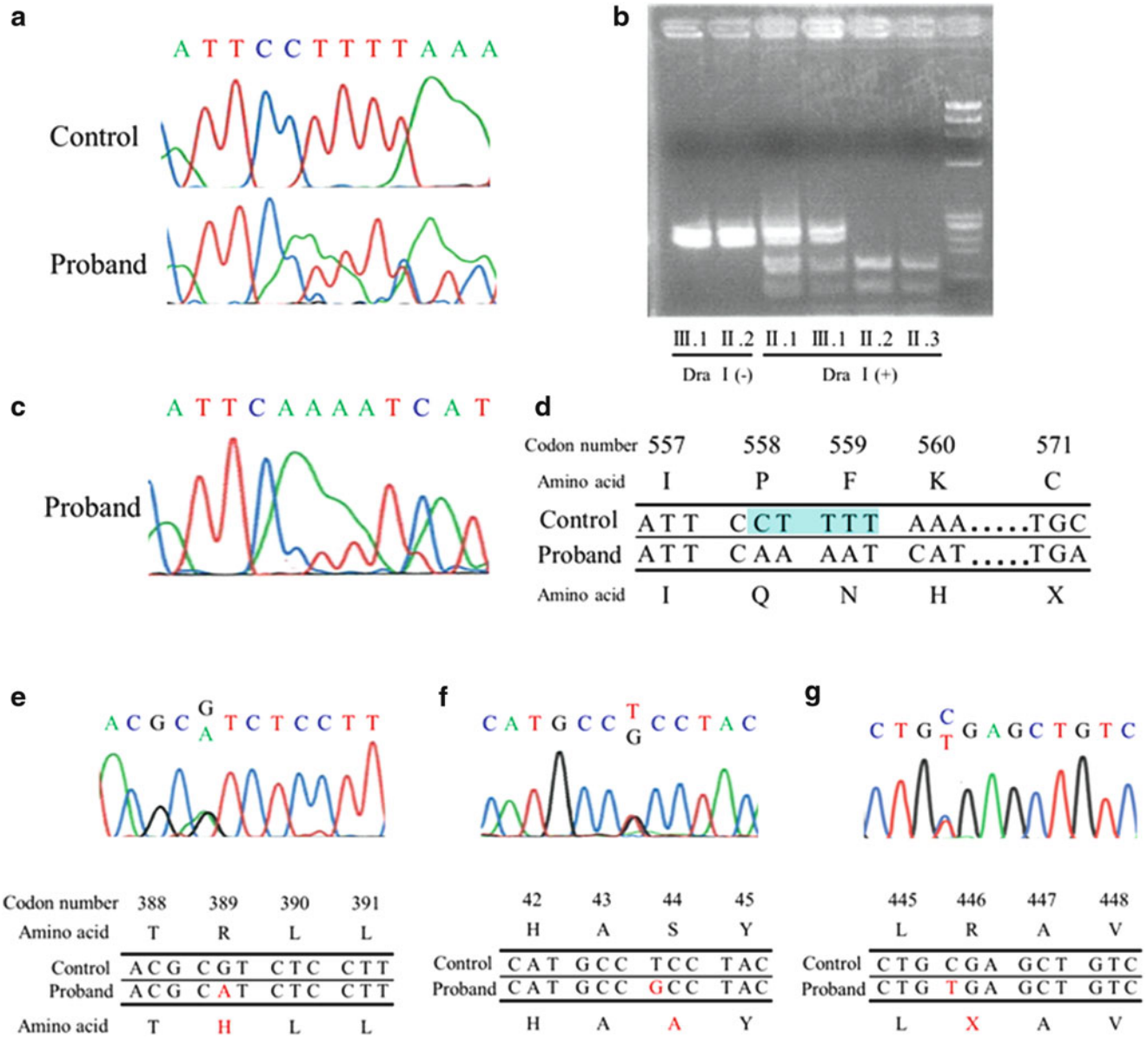

Fig. 1 DNA analysis for ABCG5 gene. (**a**) DNA sequence data of control and the proband, the latter of which showed an abnormal gap of several nucleotides in the coding region of exon 12. (**b**) Restriction enzyme analysis using DraI. Deletion of nucleotides in this region protected from fragmentation by this enzyme. (**c**) Sequencing of the non-fragmented PCR product revealed a novel mutation of a five-nucleotide deletion in exon 12 (c.1813_1817delCTTTT). (**d**) *Blue-coloured* nucleotides indicate those deleted in the proband. This deletion mutation caused a premature termination at codon 571. (**e**) DNA sequence data of the carrier which showed G to A substitution in exon 9 of the ABCG5 gene (c.1306G>A). (**f**) DNA sequence data of the carrier which showed T to C substitution in exon 1 of the ABCG5 gene (c.130T>C). (**g**) DNA sequence data of the carrier which showed T to C substitution in exon 10 of the ABCG5 gene (c.1336T>C)

Clinical and Laboratory Characteristics of Infantile Cases with Sitosterolaemia (Table 1)

We have summarised clinical and laboratory characteristics of infantile cases with sitosterolaemia of ours and those from the literature (Niu et al. 2010; Park et al. 2014; Rios et al. 2010) in Table 1, focusing on the changes in clinical manifestations before and after the weaning. Most of the infantile cases with sitosterolaemia exhibited intertriginous xanthomas associated with extremely high LDL cholesterolaemia during breastfeeding, which decreased dramatically after weaning.

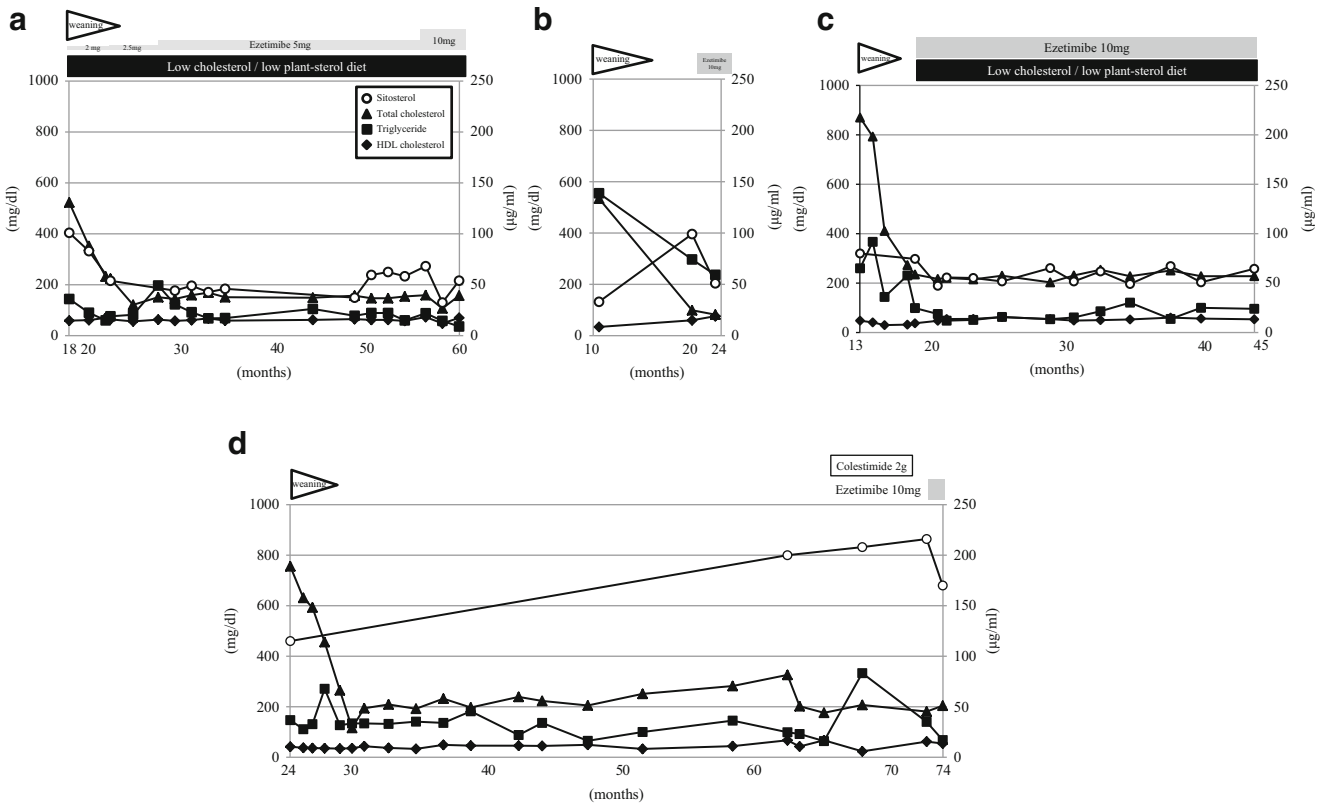

Fig. 2 Clinical courses of the cases. *Open circle* indicates sitosterol and closed triangle indicates total cholesterol. *Closed square* indicates triglyceride. *Closed rhombus* indicates high-density lipoprotein cholesterol. *Cross mark* indicates low-density lipoprotein cholesterol. Clinical courses of Cases 1 to 4 (**a**–**d**)

Discussion

The main findings of the current study are as follows: (1) we identified novel ABCG5 gene mutations (c.1813_1817delCTTTT or p.Pro558GlufsX14 and c.130T>C or p.Ser44Ala) in Japanese cases with sitosterolaemia; (2) all four infantile cases with sitosterolaemia exhibited severe hypercholesterolaemia, and three of them showed intertriginous xanthomas resembling homozygous FH; (3) weaning from breastfeeding was very effective in reducing serum cholesterol levels; and (4) ezetimibe, not colestimide, was effective in reducing serum sitosterol levels.

Interestingly, all four cases, treated in three different institutions, had been clinically misdiagnosed as homozygous FH due to severe hypercholesterolaemia and/or intertriginous xanthomas. Several subjects with sitosterolaemia, especially infantile cases, have been described as exhibiting clinical manifestations resembling homozygous FH. Thus, they were sometimes diagnosed as having "pseudo-homozygous FH" in the past (Yoshida et al. 2000). However, all four cases showed great reductions in serum cholesterol levels immediately after weaning, which would not have been observed in homozygous FH. Rios et al. reported an 11-month-old girl with compound heterozygous sitosterolaemia due to ABCG5 gene mutations who showed extreme hypercholesterolaemia (serum TC level, 1,023 mg/dl) (Rios et al. 2010). Her cholesterol levels also dramatically decreased after weaning, as with our cases. Park et al. also reported a similar case to ours, in which a 15-month-old girl whose severe hypercholesterolaemia (serum LDL-C level, 540 mg/dl) was also complicated by intertriginous xanthomas during breastfeeding; her hypercholesterolaemia disappeared two months after the introduction of a low-fat/low-cholesterol diet and cholestyramine. She was diagnosed with compound heterozygous sitosterolaemia due to an ABCG5 gene mutation (Park et al. 2014). Our findings of greatly reduced serum cholesterol levels seen after weaning in four infantile sitosterolaemic cases reinforce the previous findings (Table 1).

However, the mechanism by which serum cholesterol levels increase during breastfeeding in sitosterolaemia is still unclear. Stigmasterol, the other plant sterol that

Table 1 Summary of the clinical and laboratory characteristics of the infantile cases of sitosterolaemia reported herein plus others gleaned from the literature

Age (sex)	Ethnicity	Percutaneous xanthomas		Diet		TC (mg/dl)		LDL-C (mg/dl)		Sitosterol (μg/ml)		Mutated gene (variants)	First Author (Reference)
		Before	After	Before	After	Before	After	Before	After	Before	After		
3 months (F)	Chinese	NA	NA	Breast milk	NA	402	NA	304	NA	71	NA	ABCG5 (c.1306G>A/c.1336C>T)	Niu et al. (2010)
18 months (F)	Chinese	Present	Disappear	NA	NA	705[a]	152[b]	565[a]	71[b]	37.9[c]		ABCG5 (c.1306G>A/c.1336C>T)	Niu et al. (2010)
23 months (F)	Chinese	Present	Disappear	NA	NA	640[a]	223[c]	519[a]	NA	70.7[e]		ABCG5 (c.1306G>A/c.1306G>A)	Niu et al. (2010)
11 months (F)	Romanian	Present	NA	Breast milk 80%	NA	1023	154	837	NA	23.7	84	ABCG5 (c.47C>T/c.1336C>T)	Rios et al. (2010)
15 months (F)	Korean	Present	Disappear	Breast milk 30%	NA	675	184	540	118	NA	193.6[d]	ABCG5 (c.1336C>T/ c.904 + 1G>A)	Park et al. (2014)
18 months (F)	Japanese	Present	Disappear	Breast milk 100%	Japanese diet[e]	523	151	407	81	101	46[c]	ABCG5 (c.1306G>A/ c.1813_1817delCTTTT)	Tada (Present Case 1)
10 months (F)	Japanese	Not present	Not present	Breast milk 100%	Japanese diet[e]	555	237	NA	145	33	99	ABCG5 (c.1306G>A/ c.1813_1817delCTTTT)	Tada (Present Case 2)
13 months (F)	Japanese	Present	Disappear	Breast milk 100%	Japanese diet[e]	870	228	796	157	80	75	ABCG5 (c.1306G>A/c.130C>T)	Tada (Present Case 3)
24 months (M)	Japanese	Present	Disappear	Breast milk 100%	Japanese diet[e]	756	326	589	200	115	200	ABCG5 (c.1336C>T/ c.1813_1817delCTTTT)	Tada (Present Case 4)

TC total cholesterol, *LDL-C* low-density lipoprotein cholesterol, *Before* before weaning (during breastfeeding), *After* after weaning, *NA* not available

[a] It is not clear whether those values were affected by breastfeeding

[b] Measured under cholestyramine 1 g/day

[c] Measured under ezetimibe 10 mg/day

[d] Measured under low-saturated-fat/low-cholesterol diet

[e] Rice-based traditional Japanese food

accumulates in sitosterolaemia, inhibits the processing of a transcription factor, sterol regulatory element binding protein-2 which stimulates the transcription of hepatic LDLR (Yang et al. 2004). Accordingly, the transcription of hepatic LDLR is assumed to be inhibited, resulting in an increase in circulatory cholesterol. Breast milk contains around 100 mg/dl of sitosterol, which is independent of plant sterol intake (Laitinen et al. 2009). Thus, the infantile consumption of sitosterol is estimated at around 1,000 mg/day if an infant ingests 1,000 ml of breast milk per day; this amount of sitosterol intake is close to that of an adult but greater in terms of proportion with body weight. The accumulation of non-cholesterol sterols contributes to the very low cholesterol biosynthesis in cases with sitosterolaemia (Yang et al. 2006), leading us to speculate that severe hypercholesterolaemia observed in infantile sitosterolaemia during breastfeeding reflects the reduced catabolism of LDL or excretion of cholesterol into the bile, rather than an increase in cholesterol biosynthesis. Compared with the dietary intake of cholesterol by adults, infants ingest about 3 to 4 times the amount of cholesterol per kg body weight through breast milk (Ohta et al. 2002; Ohlsson 2010), or the high content of saturated fatty acids in breast milk (Ohlsson 2010), which may explain the extreme hypercholesterolaemia of infants with sitosterolaemia. Other possible factors could be increased absorption and retention of sterols induced by ABCG5 deficiency itself.

Niu et al. described the effectiveness of ezetimibe in reducing cholesterol levels in sitosterolaemic cases, including infantile cases (Niu et al. 2010); we noted good results with ezetimibe in the present study as well; however, our serial assessment of the clinical courses of the current cases revealed that a reduced intake of cholesterol (weaning from breastfeeding) should be considered as an important cause of the greatly reduced cholesterol levels. Although weaning was effective in reducing serum cholesterol levels and regressing intertriginous xanthomas, it was not sufficient to reduce serum levels of sitosterol. Ezetimibe, not colestimide, effectively reduced sitosterol levels by ~50%, although there is a lack of evidence for its long-term safety, especially in children. We therefore suggest weaning from breastfeeding and a restriction of plant sterols or the introduction of ezetimibe for the treatment of infantile sitosterolaemia, rather than the introduction of bile acid-sequestering resins.

A limitation of this study is that we could not elucidate the exact mechanism of the reduction of cholesterol after weaning from breastfeeding. However, it is difficult to conduct a crossover clinical trial from breastfeeding to weaning. Kinetic studies using stable isotopes could reveal a detailed record of abnormalities in lipid metabolism (Tada et al. 2012), but they are difficult to perform in infantile cases.

In conclusion, we identified two novel mutations in the ABCG5 gene in Japanese infantile sitosterolaemic cases, all of whom exhibited great reductions in serum cholesterol levels by means of weaning from breastfeeding. These novel mutations should add new support to the molecular heterogeneity of sitosterolaemia in the Asian population. Additionally, their unique response to weaning from breastfeeding, as well as to use of ezetimibe, could provide insights about the metabolic basis of sterols in humans.

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Take-Home Message

Extreme hypercholesterolaemia is exacerbated by breastfeeding in the infantile case with sitosterolaemia.

Compliance with Ethics Guidelines

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procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Informed consents were obtained from all patients or their parents (if the subject was an infant) when they visited Kanazawa University Hospital for being included in the study.

Author Contributions

Hayato Tada: designed research and performed research and wrote paper. Masa-aki Kawashiri: designed research and wrote paper. Mutsuko Takata: collected data and drafted paper. Kunihiro Matsunami: collected data and drafted paper. Atsushi Imamura: collected data and drafted paper. Misayo Matsuyama: collected data and drafted paper. Hirotake Sawada: collected data and drafted paper. Hiroyuki Nunoi: collected data and drafted paper. Tetsuo Konno: collected data and drafted paper. Kenshi Hayashi: collected data and drafted paper. Atsushi Nohara: collected data and drafted paper. Akihiro Inazu: collected data and drafted paper. Junji Kobayashi: collected data and drafted paper. Hiroshi Mabuchi: collected data and drafted paper. Masakazu Yamagishi: designed research and wrote paper.

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RESEARCH REPORT

Molecular Characterization of *QDPR* Gene in Iranian Families with BH4 Deficiency: Reporting Novel and Recurrent Mutations

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Abstract Newborn screening for PKU has been in practice in Iran since 2007. Some hyperphenylalaninemia cases have tetrahydrobiopterin (BH4) biosynthesis deficiency/disorder. Several genes including *QDPR* (encodes DHPR enzyme, the necessary cofactor for PAH activity) have been associated with the BH4. Mutations have been previously described in the *QDPR* gene. The incidence of BH4 deficiency is expected to be higher in Iran due to high rate of consanguineous marriages.

We identified a total of 93 BH4-deficient families. A multiplex set of STR markers linked to 4 genes responsible for the BH4 deficiency (i.e., *GCH1*, *PCBD1*, *PTS,* and *QDPR* genes) was used to quickly determine which gene may be responsible to cause the disease. Mutation analysis of *QDPR* gene revealed some known and novel mutations. Our findings show that no common mutation predominates, and they are scattered in the gene in our population.

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Introduction

Hyperphenylalaninemia is presented either as a result of phenylalanine hydroxylase (PAH, EC 1.14.16.1) deficiency or rarely as a form of tetrahydrobiopterin (BH_4) deficiency. It is estimated that the overall prevalence of BH4 deficiencies is about 1 in 10^6 live births in the western population, but this estimation is higher in some Mediterranean countries (Blau et al. 2001). PAH is a hepatic enzyme that needs the BH4 as a necessary cofactor for proper functioning.

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BH4 is also an essential cofactor for tyrosine and tryptophan hydroxylases, the critical enzyme in monoamine neurotransmitters (Wang et al. 2012). So BH4 deficiency can also leads to defect in neurotransmitters. As a result, BH4-deficient patients show neurological deterioration when they are only treated with phenyl restricted diet (Wang et al. 2006). So the differential diagnosis of the BH4 deficiency from phenylketonuria (PKU, MIM 261600) is very essential and should be initiated as soon as possible to reduce neurological impairment in the affected individuals.

BH4 deficiencies include heterogeneous disorders with several genes involved in the etiology of the disease. BH4 is synthesized from guanosine triphosphate (GTP) by GTP cyclohydrolase 1 (GTPCH; EC 3.5.4.16), sepiapterin reductase (SR; EC 1.1.1.153), and 6-pyruvoyl-tetrahydropterin synthase (PTPS; EC 4.3.2.12) and recycled from pterin-4-α carbinolamin by pterin-4-α-carbinolamin dehydrogenase (PCD; EC 4.2.1.96) and dihydropteridine reductase (DHPR; EC 1.6.99.7) enzymes (Blau et al. 2005). Therefore, five different genes have been identified in the synthesis or recycling of BH4.

DHPR deficiency is one form of BH4 deficiency. This deficiency is presented by psychomotor retardation, myoclonic epilepsy, microcephaly, febrile attacks, hypertonia of the trunk with limb hypertonia, and fatal course because of neurotransmitters. Patients also show defective folate metabolism which recuperate by phe-restricted diet. Diagnosis is confirmed by measuring pterins and neurotransmitter metabolites in urine and cerebrospinal fluid. Measurement of the DHPR enzyme activity in dried blood spot or fibroblast is also possible (Smith and Brenton 1996).

The mode of inheritance of the DHPR deficiency is autosomal recessive. Therefore, a higher number of patients are expected in the countries with high rate of consanguineous marriage. In this study, 93 BH4-deficient families were studied. All participants were subjected to homozygosity mapping by linked STR markers. Twenty-four families were homozygous for STR markers linked to *QDPR* locus. Full gene sequencing was done for all 24 samples.

Materials and Methods

Sample Criteria

The samples were collected from families with hyperphenylalaninemia referred to Kawsar Human Genetic Research Center and Department of Biochemistry, Pasteur Institute of Iran since 2008.

Inclusion criteria were having at least two children (either both or one being affected) born to consanguineous parents or both parents coming from the same small village and also showing autosomal recessive mode of inheritance. All families completed a questionnaire and signed informed consent.

Differential diagnosis between PAH- and BH4-deficient patients was performed according to the published diagnostic criteria (Blau et al. 2011).

Molecular Genetic Studies

Five milliliters of peripheral blood was collected from each patient, parents, and relatives (if needed) in EDTA. DNA was extracted from peripheral blood for all participants using salting-out method (Miller et al. 1988). Homozygosity mapping by the STR markers flanking the *QDPR* gene was conducted to indirectly track the probable mutated gene. Table 1 shows the STR markers used in this study and their locations, for each gene. Primer sequences are available upon request.

We carried out direct sequencing of *QDPR* gene by using specific primers amplifying seven exons and the exon/intron boundaries (Table 2).

PCR amplification was performed in a 25 μL reactions; briefly, 1.7 μL of AMS 10X buffer (Cinnagen, Tehran, Iran), 1U Taq DNA polymerase (Kawsar Biotech Co., KBC, Tehran, Iran; KBC) 0.24 μM of each primer, 0.66 μL of $MgCl_2$ (100 mM), 0.4 μL dNTP (40 mM), 12.3 μL ddH_2O, and 1.66 DMSO μL (Sigma, USA) were mixed. The PCR conditions included an initial denaturation step for 5 min at 95°C, 1 min at 95°C, 1 min at 64°C (except for exon 1 at 62°C), and 1 min in 72°C and a final extension for 10 min at 72°C for 28–30 cycles.

PCR products were directly sequenced using BigDye Terminator kit (Thermo Fisher Scientific, Life Technologies, USA, TS) according to manufacture protocol, and the samples were run on an ABI3130XL Genetic Analyzer at KBC facility. Sequences were compared with human genomic and cDNA of the *QDPR* gene, and variations were checked with NCBI reference sequence (NC_000004.12).

Result

From 93 investigated families, 24 affected probands showed homozygosity to all STR markers linked to the *QDPR* gene. This suggested high probability of disease segregation with the *QDPR* gene. All seven exons and exon/intron boundaries of the gene in the probands were sequenced, and detected mutations were confirmed in the parents. We identified different new and previously reported mutations in the *QDPR* gene (Tables 3 and 4).

Sixteen different types of mutations were found. Ten of them had not been reported previously. Most of the mutations

Table 1 STR markers used this study

STR marker	Location	Repeated seq	Length of PCR product
D4QDPRSD0. 2	Chr4:17464963–17465289	TATC	327
D4QDPRSD2	Chr4:17281517–17281740	CTAT	224
D4SQDPRSD9. 6	Chr4:16519399–16519589	ATCC	191
D4QDPRSD10	Chr4:16483281–16483592	TAGA	312
D4QDPRSU13	Chr4:18845242–18845393	GATA	129–162
D4QDPRSU17	Chr4:19234187–19234517	TATAGA	314–342

Table 2 List of QDPR exon amplification primers and product sizes (bp)

Exon number	Primer sequences	Primer length (bp)	Product size (bp)
1	F: 5′-TTACACTTCACAAATTAATGCTCGT-3′ R: 5′-AAACAGGAATAGACGCGTAGACC-3′	25 23	605
2	F: 5′-CCCTCATTTCTATGTGTGACTCTT-3′ R: 5′-CAAAGGAAGAACATACAGCCAG-3′	24 24	247
3	F: 5′-TCTTCCGTCTAATTCTCAAAGC-3′ R: 5′-GTGTATATCCCGGAATCTTTACA-3′	22 23	363
4	F: 5′-TGTGCTGTTTGTGTTAGACCTTG-3′ R: 5′-ATCTATCTGTTAAGCAGCTTAGAGG-3′	23 25	408
5	F: 5′-GAGGAGGCCAGATGCAGCTA-3′ R: 5′-GTGGAAAGCTACAGTCAGACAAAC-3′	20 24	315
6	F: 5′-GTGCCAGAGGCTCTAGGTTGTC-3′ R: 5′-CGGAATCTCAGAGTAGCTGGACT-3′	22 23	377
7	F: 5′-GTGCCAGAGGCTCTAGGTTGTC-3′ R: 5′-AGTTAACAGAGATCAACGGATGC-3′	22 23	412

Table 3 Types of mutation, amino acids, and number seen for previously reported mutation (NM_000320)

Codon and nucleotide changes	c.DNA change	Amino acid change	No. seen	Genotype	References
Cd18GGC>GAC	c.[233G>A];[233G>A]	p. [(Gly18Asp)]	4	Homozygous	Scrutton et al. (1990)
Cd 23GGT>GAT	c.[248G>A];[248G>A]	p. [(Gly23Asp)]	1	Homozygous	Romstad et al. (2000)
Cd150TAC>TGC	c.[629A>G];[629A>G]	p. [(Tyr150Cys)]	3	Homozygous	(Dianzani et al. 1993)
Cd221CGA>TGA	c.[661C>T];[661C>T]	*p.(R221*)*	2	Homozygous	Smooker and Cotton (1995)
Cd158CAC>TAC	c.[652C>T];[652C>T]	p. [(His158Tyr)]	1	Homozygous	Smooker et al. (1993)
Cd97DelC	c.472delC	–	1	Homozygous	Romstad et al. (2000)

were missense mutations and only two were homozygous deletion of a nucleotide. We also found two nonsense mutations. From 12 missense mutations, two would cause termination of codon. Two different types of mutations were seen for codon 115 (exon 4). One creates a termination codon and the other causes substitution of leucine to serine. p. [(Gly18Asp)] was found to be the most common mutation which was seen in four different families. Types and frequencies of mutations are shown in Tables 3 and 4.

The pathogenicity of identified mutations were checked in related websites such as Polyphen-2 (Adzhubei et al. 2010), SIFT (Kumar et al. 2009), and also HOPE project (Venselaar et al. 2010). Results and suggested effects are shown in Table 4.

Table 4 Novel mutations identified in this study and related information (NM_000320)

Codon and nucleotide changes	Type of mutation	Exon	Amino acid change	cDNA change	No. seen	SIFT suggestion	Polyphen suggestion
Cd115 TCG>TAG	Nonsense	4	*p.(S115*)*	c.[344C>A]; [344C>A]	2	Tolerated	–
Cd17 GGC>TGC	Missense	1	p. [(Gly17Cys)]	c.[49G>T]; [49G>T]	1	Damaging	Probably damaging sensitivity: 0.00; specificity: 1.00
Cd217 del A	Deletion	7	–	c.649 del A	1	–	–
Cd185 CTG>CCG	Missense	6	p. [(Leu185Pro)]	c.[554 T>C]; [554 T>C]	1	Damaging	Probably damaging sensitivity: 0.00; specificity: 1.00
Cd89 GGA>AGA	Missense	3	p. [(Gly89Arg)]	c.[265G>A]; [265G>A]	1	Damaging	Probably damaging sensitivity: 0.00; specificity: 1.00
Cd 115 TCG>TTG	Missense	4	p. [(Ser 115Leu)]	c.[344C>T]; [344C>T]	1	Tolerated	Probably damaging sensitivity: 0.00; specificity: 1.00
Cd225 GGA>AGA	Missense	7	p. [(Gly225Arg)]	c.[673G>A]; [673G>A]	1	Damaging	Probably damaging sensitivity: 0.00; specificity: 1.00
Cd 237 ACG>ATG	Missense	7	p. [(Thr237Met)]	c.[710C>T]; [710C>T]	1	Damaging	Probably damaging sensitivity: 0.00; specificity: 1.00
Cd 114 ACA>ATA	Missense	4	p. [(Thr114Ile)]	c.[341C>T]; [341C>T]	2	Damaging Score: 0.02	Probably damaging score: 0.998; sensitivity: 0.27; specificity: 0.99
Cd 163 AGC>AAC	Missense	5	p. [(Ser163Asn)]	c.[488G>A]; [488G>A]	1	Damaging Score: 0.01	Possibly damaging score: 0.885 sensitivity: 0.82; specificity: 0.94

Discussion

Frequency of PKU in Iran has been estimated to be about one per 5000 live births (Setoodeh et al. 2015). The above study and also data obtained at the molecular level show that approximately 3–5 of the hyperphenylalaninemia cases ended up being BH4. This number is about 3–5 per 100,000 live births. This number is higher than 1 in 10^6 reported in some Mediterranean countries (Blau et al. 2001).

We found that most of the mutations (10 out of 16) were novel, perhaps because of disease rarity in other populations: eight missense, one nonsense, and one single nucleotide deletion mutations. The *p. (S115*)* mutation changes serine to a stop codon in exon 4. This causes a truncated protein that lacks the active site of the enzyme. The active site of the enzyme, which is also a proton acceptor site, is situated on the 150th amino acid. As a result of this mutation, protein synthesis stops before reaching the active site. It can be postulated that the enzyme should have no or very little activity. However, SIFT predicted that this mutation can be tolerated. However, we can argue that the phenotype of the affected child is more aligned with our claim.

DHPR has a NADH binding fold at the N-terminus formed by two β-sheets and an α-helix, named the 13c43-fold or the "Rossmann fold" (Scrutton et al. 1990). Within this fold, there is a motif that contains three highly conserved glycine residues in the order of Gly-X-Gly-X-X-GIy. These glycines are positioned in codons 18, 20, and 23. Two mutations have already been reported in these codons (Romstad et al. 2000), and we found them again in five Iranian families. We found a novel mutation in codon 17: p. [(Gly17Cys)] (Table 4). This mutation substitutes a hydrophilic amino acid cysteine with neutral glycine. This residue is 100% conserved, and its substitution can disturb the multimeric interactions and affect the catalytic activity. Therefore, this change has a high potency for being pathogenic. The result of Polyphen2 and SIFT also confirms this (Kumar et al. 2009; Adzhubei et al. 2010; Venselaar et al. 2010).

Substitution of p. [(Gly23Asp)] (Table 3) has been found to be a frequent mutation in patients of Mediterranean origin (Smooker et al. 1993). We did not see this mutation. However, we observed that p. [(Gly18Asp)] mutation was more frequent in the Iranian population. The mentioned mutation had been reported in a Turkish patient with severe phenotype (Romstad et al. 2000).

Four amino acid substitutions, namely, p. [(Gly17Cys)], p. [(Leu185Pro)], p. [(Gly225Arg)], and p. [(Thr237Met)], are potentially pathogenic since they are not present in the single nucleotide polymorphism database (Exome Variant Server, http://evs.gs.washington.edu/EVS/). This was supported by bioinformatics predictions by Polyphen-2 (Adzhubei et al. 2010) which indicated that all of these are

Table 5 Distribution of different abnormalities in PTPS- and DHPR-deficient patients

	Mean age at diagnosis	CNS abnormality	Convulsions	Abnormal movement	Impaired tonus	No symptoms	Retardation
PTPS	2 month	9	12	4	15	4	16
%	–	30	40	13.3	50	13.3	53
DHPR	2 month	11	20	3	24	6	20
%	–	45.8	83	12.5	100	25	83

"probably damaging" (score 1.00), and SIFT (Kumar et al. 2009) indicated that they may be "damaging" (score 0).

Regarding the p. [(Leu185Pro)] mutation, proline is a very rigid residue that might abolish the required flexibility of the protein at the highly conserved position 185. Since proline residue is smaller than leucine, this will cause a possible loss of external interactions and prevents multimeric contacts. The 225th amino acid has been shown to be 100% conserved residue (Venselaar et al. 2010), and it would damage the protein if it is replaced with an arginine which is a bigger amino acid with different charge. Also, methionine in comparison with threonine at residue 237 shows differences in hydrophobicity. Therefore, it is expected that this substitution affects hydrogen bond formation. This threonine residue is much conserved and also methionine is a bigger residue; therefore, it is expected that it cannot be buried in the core of the protein. In conclusion, based on the HOPE project's (Venselaar et al. 2010) information, these changes are possibly damaging to the protein.

Also, other mutations including p. [(Gly89Arg)], p. [(Ser 115Leu)], p. [(Thr114Ile)], p. [(His158Tyr)], and p. [(Ser163Asn)] are potentially pathogenic because they have not been reported in the single nucleotide polymorphism database (Exome Variant Server). Moreover, bioinformatics prediction by Polyphen-2 and SIFT indicated that these changes are either "probably damaging" or "damaging" with different scores (see Table 4). The p.[(Gly89Arg)] amino acid substitution introduces a bigger and a less hydrophobic residue at this position that can disturb the multimeric interactions. At the 89th amino acid, only glycine is flexible enough to make torsion angles, so mutation into arginine will force the local backbone into an incorrect conformation which may disturb the local structure. At 115th, wild-type residue is a highly conserved glycine that forms a hydrogen bond with the serine on 111th position. Therefore, mutation in this site will cause loss of hydrogen bonds in the core of the protein and as a result disturbs correct folding. Besides, at this position the mutant leucine residue is bigger than serine and probably will not be buried in the core of the protein which is important for the main activity of the protein.

By substitution of threonine with isoleucine at amino acid 114, the multimeric contacts would be disturbed. Since the mutated isoleucine is located in a domain that is important for the main activity of the protein, differences between the wild-type and mutant residue can damage the core structure of this important domain and thereby affect the catalytic activity. The histidine at position 158 is a conserved residue and is buried in the core of the protein. The mutant tyrosine residue is bigger and probably will not fit in the space neatly. Also, because of different hydrophobicity, the mutation p.H158Y will cause loss of hydrogen bonds in the core of the protein and as a result disturbs correct folding.

Finally, mutation p. [(Ser163Asn)] changes serine into asparagine at position 163 which can damage the protein. One reason to support this claim is that the wild type is highly conserved. Serine forms a hydrogen bond with the glutamine on position 159, so because of the size difference, the mutant residue cannot make the same hydrogen bond as the original wild-type residue. The mutant asparagine residue is bigger with less hydrophobicity, so it can disturb interactions with other molecules or other parts of the protein which results in a defective protein.

Regarding genotype/phenotype correlation in our patients, we conclude that no clear correlation was observed between various mutations and also the type of the gene involved. One reason could be due to the age of admission, age at treatment, prior management by other physicians, parental adherence to the physicians' orders, etc. (see Table 5). Molecular analysis of the remaining families is ongoing.

Number of recurrent mutations found was limited in the studied population. This may be due to either small sample size or the heterogeneity of Iranian population. Since BH4 deficiency is a rare disease, therefore, 24 samples may not be few. This finding may also be supported by a suggestion (Blau et al. 2001) that recurrent mutations, in unrelated individuals with BH4 deficiency, are more likely to be due to recurrent mutations at the CpG dinucleotide rather than founder effects. Multi-founder effect hypothesis may also be valid in large and heterogeneous populations like Iran, with several ethnicities and long history of civilization and human migrations (Najmabadi et al. 2003).

Take-Home Message of the Article

QDPR accounts for the majority of BH4 deficiency with a variety of mutations in Iran.

Compliance with Ethics Guidelines

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Contribution of Authors in Project

Maryam Abiri: Interpretation of data and drafting of manuscript

Hannaneh Foroozani: Data collection and doing molecular genetic testing in laboratory

Shadab Salehpour: Clinical diagnosis of patients and responding for clinical comments of the reviewers

Hamideh Bagherian: Genetic counselor of medical genetics laboratory of Kawsar Human Genetics Research Center

Zohreh Sharifi: Primer designing

Shohreh Khatami: Performing biochemical tests for patients and analysis of data

Mohammad Reza Alaei, Aria Setoodeh, Farzaneh Rohani: Clinical diagnosis of patients

Leyli Rejali: Sequencing of pcr product

Sara Azadmehr: Doing molecular genetic testing in laboratory

Sirous Zeinali: Supervisor of the project and edit of manuscript

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RESEARCH REPORT

Erratum to: Glutaric Acidemia Type 1-Clinico-Molecular Profile and Novel Mutations in *GCDH* Gene in Indian Patients

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The author name **Puneet Kaur** was misspelled in the chapter. The correct spelling is **Punit Kaur**

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